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THE EFFECT OF MIREX AND CARBOFURAN ON ESTUARINE
MICROORGANISMS

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THE EFFECT OF MIREX AND CARBOFURAN ON ESTUARINE MICROORGANISMS

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ABSTRACT

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It was concluded that neither mirex nor carbofuran would have a deleterious effect on estuarine bacteria under normal conditions, and there was no evidence of bioaccumulation. Degradation products of both compounds were shown to be toxic to some microorganisms.

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SECTION I

CONCLUSIONS

It is concluded from this study that: (1) essentially all of the mirex in aqueous solution will be removed by adsorption to particulate matter (clay and dead bacterial cells) and will resist degradation; (2) only a small percentage ($\sim 15\%$) of carbofuran will be removed from aqueous solution by adsorption but appreciable hydrolysis will occur; (3) there was no evidence of bioaccumulation of either mirex or carbofuran; (4) a wide variety of physiological types of bacteria studied in both pure culture and in microcosm systems were not adversely affected by mirex or carbofuran in concentrations up to 100 ppm although both compounds inhibited primary productivity and (5) a variety of degradation products inhibit a number of different microorganisms and processes (particularly nitrification and primary productivity) at concentrations as low as 1 ppm, but it is considered unlikely that these compounds would ever reach the 1 ppm concentration in the estuarine environment.

Based upon the data obtained in this investigation it is concluded that mirex and carbofuran in concentrations below 100 ppm would not have a deleterious effect on estuarine bacteria.

SECTION II

RECOMMENDATIONS

It is recommended that studies be conducted to identify the microbiologically toxic material(s) occurring in some batches of mirex as well as the products resulting from UV radiation of moist mirex.

The toxicological and chemical behavior of conjugates of carbofuran metabolites should be performed to completely delineate the effect of this pesticide.

Since mirex and probably carbofuran will partition into an organic phase from an aqueous system and because oil is a common contaminant in estuaries, it is recommended that the impact of oil-mirex and oil-carbofuran systems should be examined for their deleterious impact on estuarine organisms, biodegradability and chemical and physical fate

These investigations should be followed by studies of more complex systems which would include higher members of the food chain, both in the laboratory and in the field studies.

SECTION III

INTRODUCTION

The primary utilization of mirex as an insecticide is in the control of the western harvester and the imported fire-ant. The latter species infest most of the southeastern United States and millions of acres are treated annually in this area by aerial application of baits containing this toxicant at a rate of 0.20 to 1.66 g per hectare. There are few areas in the southeast that do not have some history of mirex application.

Carbofuran is registered for utilization on field corn, rice, peanuts, alfalfa and sugar cane (EPA¹). It is applied to and incorporated into the soil at rates from 45.9 to 1725.5 g per hectare.

With the history and rate of application of these pesticides to the greater part of the southeast, it is obvious that some portion, both of mirex and carbofuran, will enter the estuaries of the Gulf of Mexico. In these estuaries, microorganisms are a vital link in the overall ecological system. There are no data available on the effect of either mirex or carbofuran on this estuarine microbial ecosystem.

In order to fill this gap in our existing knowledge, the Environmental Protection Agency awarded Mississippi State University Contract No. 68-03-0288, "The Effect of Mirex and Carbofuran on Estuarine Microorganisms".

The objective of this investigation was to supply a sufficient amount of scientifically valid data on the fate and effects of mirex and carbofuran in the estuary in such a form that these data can be employed in deriving water quality standards for these pesticides. Specifically, the work was designed to establish the chemical, physical and microbiological fate of these pesticides in the estuarine environment and determine meaningful effect(s) on important estuarine microorganisms and their activities. It is extremely important to maximize the transferability of scientific results to the environment, and thus the experimental approach described involves the use of microcosm systems as well as pure culture studies.

SECTION IV

MATERIALS AND METHODS

MATERIALS

Carbofuran

Technical carbofuran (98% purity) and analytical standards (99% purity) were obtained from FMC Corporation. Analytical standards of 3-hydroxy- and 3-ketocarbofuran as well as the three phenol metabolites were obtained from the same source. Technical carbofuran was further purified by recrystallization from toluene in one case, and ethanol in another. (See Figure 1)

Mirex

One batch of mirex (batch 1) was obtained from the FDA Laboratory at Perrine, Florida. All other batches of mirex (technical) were obtained from Allied Chemical Company. This material was purified by chromatography on a silica gel column with cyclohexane as the solvent. Examination of the technical material and some analytical standards showed the presence of hexachlorobenzene and other less abundant compounds of similar volatility. Hexachlorocyclopentadiene was usually present in very small quantities; and, while the other compounds could not be identified, it was shown that they were not hexachlorofulvene or 1,2,3,4,5-pentachloro-5-trichloromethyl cyclopentadiene. The total of these impurities was estimated to be about 0.1%. The chromatographic purification effectively removed these impurities when only the early eluting fractions were utilized.

The major product of the pyrolysis of mirex is hexachlorobenzene (Kennedy *et al.*²), so the presence of this impurity is not surprising.

Kepone

Technical kepone was obtained from Allied Chemical Company and purified by azeotropic distillation of water and crystallization from benzene.

Mirex and Kepone Photoproducts

1,2,3,4,5,5,6,7,9,10,10 undecachloro [5.3.0.0².6.0³.9.0⁴.8] pentacyclodecane, **1**, 1,3,4,5,5,6,7,9,10,10-decachloro [5.3.0.0².6.0³.9.0⁴.8] pentacyclodecane **2**, 1,2,3,4,5,5,6,7,8,9,10 undecachloro [5.3.0.0².6.0³.9.0⁴.8] pentacyclodecane **3** and 1,2,3,4,5,6,7,8,9,10-decachloro [5.3.0.0².6.0³.9.0⁴.8] pen-

tacyclodecane, **4** were prepared from mirex by photochemical methods (Alley *et al.*^{3, 4}). 1,2,3,4,6,7,8,9-, 10,10 decachloro-5 hydroxy-[5.3.0.0^{2,6}.0^{3,9}.0^{4,8}] pentacyclodecane, **5** was prepared by the method of Dilling⁵. (See Figure 2)

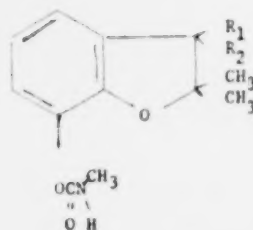
Medium

Unless otherwise noted, all media used in this study were obtained from Difco Laboratories and prepared with Rila sea salts mixture to obtain 15 ppt salinity. The mineral salts medium contained 1.0 g KNO₃; 0.5 g K₂HPO₄·3H₂O; 0.2 g MgSO₄·7H₂O; and 0.05 g FeCl₃·6H₂O per 1000 ml distilled water (final pH adjusted to 7.0).

Cultures

Pure cultures of estuarine bacteria were obtained from the stock collection at Gulf Coast Research Laboratory, Ocean Springs, Mississippi. *Methanomonas methanooxidans* (Brown and Strawinski), *Mycobacterium*, and *Nocardia* were obtained from stock collection at Mississippi State University. Sediments used for the study of methane consumption and nitrification were obtained from Biloxi Bay, Mississippi.

Figure 1. Carbofuran and derivatives

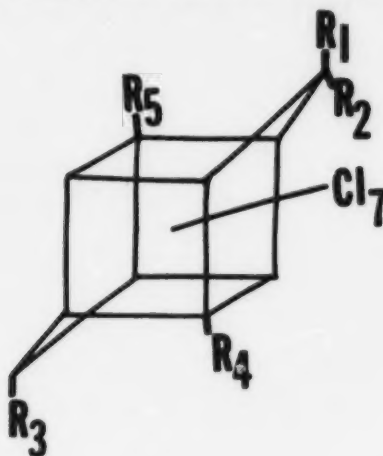


Carbofuran, R₁ = R₂ = H

3-hydroxycarbofuran, R₁ = H, R₂ = OH

3-ketocarbofuran, R₁, R₂ = O

Figure 2. Structural configuration of mirex and derivatives of mirex.



For mirex, $R_1 = R_2 = R_3 = R_4 = R_5 = \text{Cl}$

For kepone, $R + R = \text{double bond oxygen}$; $R_3 = R_4 = R_5 = \text{Cl}$

For 8-hydrogen derivative, 1, $R_4 = \text{H}$; $R_1 = R_2 = R_3 = R_5 = \text{Cl}$

For 2,8-dihydrogen derivative, 2, $R_4 = R_5 = \text{H}$; $R_1 = R_2 = R_3 = \text{Cl}$

For 10-hydrogen derivative, 3, $R_1 = \text{H}$; $R_2 = R_3 = R_4 = R_5 = \text{Cl}$

For 5,10-dihydrogen derivative, 4, $R_1 = R_3 = \text{H}$; $R_2 = R_4 = R_5 = \text{Cl}$

For reduced kepone, 5, $R_3 = \text{OH}$; $R_1 = R_2 = R_4 = R_5 = \text{Cl}$

METHODS

Chemical Methods

Analysis of Carbofuran and its Derivatives-

A number of problems was encountered in attempts to develop good analytical methods for these compounds. Direct analysis by gas chromatography was extensively studied utilizing a conductivity detector that is specific for nitrogen. Despite numerous approaches that were tried, decomposition during the analysis turned out to be an insurmountable problem.

Direct determination by ultraviolet absorption (Metcalf, *et al.*⁶) was not suitable for the hydrolysis studies because it was shown that the absorption observed by these workers was not due to either the carbofuran or any of its identified derivatives, including the phenol.

Several other methods were tried and failed, but two methods were found to provide satisfactory results. The gas-liquid chromatography (glc) method is most suitable for low levels and where carbofuran and its 3-hydroxy- and 3-keto-derivatives are expected to occur together. The high pressure liquid chromatography (hplc) method is best suited for higher concentrations and where only the carbofuran is expected to occur along with its phenolic hydrolysis product.

Analysis of Carbofuran in Water by hplc-

Analyses were performed directly on the aqueous solutions by injecting the samples into a 1.8 m by 1.5 mm i.d. column packed with Corasil II. The packing was deactivated by equilibrating it with diisopropyl ether saturated with water and the same solvent system was used for the mobile phase. Carbaryl was used as an internal standard and the quantitation done by measuring peak heights.

Analysis of Carbofuran in Sediments-

Ten grams of sediment and water were shaken with 50 ml of methylene chloride in a centrifuge bottle. The sediments were removed by centrifugation and filtration through glass wool. The sediment was treated twice more with 25 ml of methylene chloride. The combined extracts were placed in a separatory funnel, and the methylene chloride layer was removed. The aqueous phase was washed with 25 ml of methylene chloride, the combined extracts dried with sodium sulfate and the solvent removed with a Kuderna-Danish evaporator. The residue was taken to dryness under a stream of nitrogen and then redissolved in 5 ml of 15% diethyl ether in petroleum ether. The entire sample was introduced into a 1 cm diameter column containing 4 g of activated florisil which had been prepared with 10 ml of petroleum ether. The column was eluted with 70 ml of 15% diethyl ether in petroleum ether followed by 100 ml of 50% diethyl ether. The 50% fraction was reduced to a small volume and transferred to a Miniaktor (Applied Science Laboratories, Inc.) and the solvents removed with a stream of nitrogen. Ethyl acetate (0.2 ml) and heptafluorobutyric anhydride (0.1 ml) were added to the reactor, and it was tightly capped, wrapped with aluminum foil and heated at 45 C for 16 h. The contents were diluted with 10 ml of petroleum ether, washed three times with water, and the organic layer dried and made to an exact volume for electron capture gas chromatographic analysis. The parameters for this analysis were: a 1.5 m by 4 mm i.d. all glass column utilizing on-column injection was used; the column temperature was 165 C, the injector 245 C, and the detector 210 C. The column was packed with 3% diethylene glycol succinate on Gas-Chrom Q. With these conditions, carbofuran had a retention time of 1.2 min, 3-hydroxycarbofuran 1.8 min, and 3-ketocarbofuran 2.4 min.

Analysis of Mirex and its Derivatives-

Mirex and the photoproducts described in the materials section can all be quantitated by the following procedures.

Analysis of Mirex in Water-

A sample of the water to be analyzed was extracted three times with 75 ml portions of petroleum ether. The combined extracts were dried and passed through a florisil column. Petroleum ether was used to elute the column and the eluate concentrated to 1-10 ml and then quantitated by electron capture gas chromatography. This method was shown to produce high recoveries (90-100%) of the compounds.

Analysis of Mirex in Sediments-

Sediments were analyzed in the same manner as waters except that the samples were shaken with two volumes of 1:1 acetone-petroleum ether. The samples then were centrifuged and the superna-

tant decanted into a 1 liter separatory funnel. The petroleum ether layer was washed twice with water, the organic layer dried and introduced into a florisil column. After this point in the procedure, the water method was followed exactly.

Other Chemical Methods-

Nitrate, nitrite and ammonia were determined by standard procedures (APHA⁷). Protein-nitrogen was determined according to the modification of Lowry's procedure (Cook⁸).

Microbiological Methods

Disc Assay Techniques-

A number of variations of the disc assay procedure was employed to test the effect of mirex on bacteria. Tests involved dissolving test compounds in acetone and placing the material on number 740E, 12.7-mm paper discs (Scheicher and Schnell, Inc., Keene, N.H.). The saturated discs were kept overnight to allow the acetone to evaporate. In other tests, solid discs of test compounds were prepared by using an hydraulic press with a 12.7-mm die and $137,880,000 \text{ N/m}^2$ ($14,062,000 \text{ kg/m}^2$; $1 \text{ kg} = 9,805 \text{ newtons}$) pressure. In other tests, loose particles of test compounds were applied directly on the surface. In all cases, seeded nutrient agar plates were employed with incubation at 30 C for 24 h.

Growth Curve Studies-

Nutrient broth medium was employed and growth was monitored by plate count or changes in optical density of the broth using a B&L Spectronic-20 colorimeter (520 nm). Studies were conducted in duplicate at 30 C for 57 h.

Qualitative Physiological Studies-

Qualitative observations of test-tube reactions were employed to evaluate the effect of varying concentrations of test compounds on glucose fermentation and hydrogen sulfide production. Glucose fermentation studies were conducted in Durham fermentation tubes using 1% glucose broth with phenol red indicator. Lead acetate semisolid agar was employed to test for hydrogen sulfide production. For these tests, the compounds were added aseptically to the test media after sterilization. Incubation was at 30 C for 5 days.

Bacto-gelatin agar, Bacto-spirit blue agar with cottonseed oil, Bacto-starch agar, skim milk, and finely ground purified chitin in $\frac{1}{2}$ strength Bacto-marine agar were employed for the studies on gelatin-, lipid-, starch-, casein-, and chitin-hydrolysis, respectively. Test compounds were applied to the plates using three different techniques: (1) test compounds (in acetone) were spread over the entire surface of the agar, the acetone was allowed to evaporate, and the test bacteria were streaked in a single line across the surface; (2) the test compounds (in acetone) were applied in a single line on the surface of the agar and the test culture was streaked evenly over the entire plate; and (3) filter paper strips impregnated with test compounds were placed in the agar for streak plates or on the surface of pour plates.

Quantitative Physiological Studies -

Tests on the consumption of methane by estuarine sediments were conducted in Sohngen units as modified by Hutton and ZoBell⁹. The inoculum was prepared by making a slurry of 1000 g of sediment and 3000 ml of 15 ppt salinity mineral salts medium (prepared with Rila sea salts). Aliquots of 50 ml of the slurry containing 0.0, 0.1, 1.0, 10.0, or 100.0 ppm of the test compounds were placed in the "reactors". The air atmosphere of the "reactors" was replaced with a gas mixture consisting of 65% CH₄, 30% O₂, and 5% CO₂. Incubation was carried out at 30 C under static conditions. Gas consumption was measured daily in terms of millimeter increase in height of the liquid in the "reactors".

Experiments employing Sohngen units for the studies utilizing *M. methanooxidans* were conducted in accord with the procedures described by Brown *et al*¹⁰. Appropriate concentrations (same as in sediments above) of filter-sterilized test compounds in acetone were added to empty "reactor" bottles, and the acetone was allowed to evaporate prior to the addition of the mineral salts medium. After gassing, the "reactors" were inoculated with 3 ml of a culture of *M. methanooxidans*, and the units were incubated at 30 C under static conditions.

Medium for the studies on ammonification was peptone broth containing Rila sea salts mixture to yield 15 ppt salinity. The culture vessels were 500 ml Erlenmeyer flasks each containing 300 ml of medium. Filter-sterilized test compounds were added to the flasks so that the final medium contained 100 ppm. The inoculum consisted of 1 ml of a 24 h broth culture of the test organism, and incubation was carried out at 30 C for 5 days under static conditions, in duplicate. Optical density and ammoniacal-N were measured daily. Protein was determined on the filtered (0.45 μ membrane filter) medium initially and at the termination of the experiment.

Experiments on nitrate reduction were conducted in duplicate in 500 ml Erlenmeyer flasks each containing 300 ml of Bacto-nitrate broth. The concentrations of test compounds in these tests were 0, 1, 10, and 100 ppm. Each flask was inoculated with 1 ml of a 24 h broth culture of *Achromobacter* #274 and incubated at 30 C under static conditions. Optical density, NO₃-N, and NO₂-N were determined initially and thereafter at 6 to 12 h intervals up to 48 h.

Nitrification studies were performed in 500 ml Erlenmeyer flasks each containing 300 ml of ammonium-calcium carbonate medium at a salinity of 15 ppt. Test compound concentrations were 0, 1, 10, and 100 ppm. The inoculum was 5 ml of a mixture of one part estuarine sediment to three parts mineral salts medium at a salinity of 15 ppt. All tests were conducted in duplicate, and incubation was carried out under shake conditions at ambient temperature. Substrate depletion (NH₃) and product formations (NO₃, NO₂) were determined initially and every 5 days thereafter up to 20 days.

Primary productivity studies were conducted utilizing estuarine pond water directly or after enrichment of the phytoplankton population by incubation in the presence of added nitrate, phosphate and light for 12 h. Test compounds (contained in acetone) were pipetted into each 300 ml B.O.D. bottle and 10 ml of boiling distilled water added immediately. The bottles then were filled with the test water and incubated at ambient temperature for 12 h between 2 sets of fluorescent lights, each having 8 light bulbs. Bottles used for the dark reaction were painted black and covered with aluminum foil. Solvent controls and controls containing 1000 ppm HgCl₂ were included in the tests. Dissolved oxygen (D.O.) was determined with an Oxygen Meter Model YSI54.

Studies on Chemotaxis -

A gram negative rod-shaped motile bacterium was isolated from an estuarine environment (Bay St. Louis, MS) on nutrient agar increased to 15 ppt salinity.

The isolate was inoculated into an Erlenmeyer flask containing Bacto-nutrient broth having a salinity of 7.5 ppt and incubated at ambient temperature under shake conditions for 15 to 18 h. Cells were harvested by centrifugation at 20 C at 4500 rpm for 10 min, washed 3 times in physiological saline, and resuspended to the desired concentration in a solution having a salinity of 7.5 ppt. Acetone containing mirex or carbofuran at 10 ppm or 50 ppm was pulled into capillary tubes by capillary action until the tubes were $\frac{3}{4}$ full. The tubes were then placed in an oven at 80 C, thus leaving a residue of the pesticide on the internal walls of the capillary tube. Acetone "residue" tubes were prepared in a similar manner except no pesticide was contained in the acetone.

Chemotaxis was determined by use of the capillary tube technique, and the procedures employed were a modification of those described by Mitchell, Fogel and Chet¹¹.

Capillary tubes were filled $\frac{3}{4}$ full with the desired test solution; subsequently the top ends of the tubes were plugged with silicone grease and the outsides of the tubes were washed in a stream of water and wiped dry with a sterile Kimwipe®. The tube was inserted into a small vial containing 0.4 ml cell suspension of approximately 3.0×10^8 viable cells/ml for 12 min. Subsequently, the tube was removed, washed in a small stream of water and broken into 3 or 4 sections (excluding upper $\frac{1}{4}$ section) into physiological saline diluent. Dilutions in physiological saline were prepared, plated, and spread on the surface of nutrient agar plates containing 7.5 ppt salinity.

Microcosm Studies-

Microcosms consisted of all glass vessels (usually 4 liter wide mouth jars) containing a mixture of estuarine sediment and sea water. This mixture was prepared by homogenizing sediment from the top 11.6 cm of the estuary bottom with artificial sea water in a ratio of 2:1 (w/w) using a large Waring blender. Within 24 h after the sediment mixture was placed in the vessel, settling occurred and about a 3 cm deep layer of clear water appeared over about 9 cm of sediment, with the top sediment layer oxidized and the bottom layer reduced as indicated by the color. The microcosm thus mimicked the estuarine sediment system. Some infauna forms of polychaetes survived the homogenizing and became active in the microcosms.

Sediments in these studies were obtained from Davis Bay and were generally described as "sandy course silt". Sediments were checked for mirex and it was not detected.

A single lot of technical grade carbofuran (Niagara Chemical Division, FMC Corporation, Middleport, N.Y., ME-L514, 4717-54-A, 8/9/73) was used for all the sediment microcosm studies. Two lots of technical grade mirex were used with lot number SN-16-6988-43 (Allied Chemical Corp., Baltimore, Md.) being used for studies on the effect of various mirex concentrations on the heterotrophic bacterial populations and the various biochemical groups of organisms. Lot number SN-3-100-36 (Mirex Technical, Allied Chemical Corp., Baltimore, Md.) was used for the temperature-salinity studies and for all other studies. The purified mirex and carbofuran were prepared by recrystallization, and the water washed carbofuran prepared by placing in a filter funnel and washing with distilled water until the filtrate was colorless.

Pesticides in concentrations of 1 ppm and greater were added as the dry powder by weight during the sediment mixing procedure. The amount added thus represented the addition to the whole system. However, after the microcosm settled, most of the pesticides were probably in the sediment. For a pesticide concentration of 0.1 ppm, 10 mg of the pesticide were dissolved in 100 ml of acetone and 0.1 ml of this solution added to each 1000 g of the sediment water mixture.

Rila sea salts dissolved in distilled water was used in all studies at a salinity of 15 ppt unless otherwise noted.

Microcosms were incubated in the dark at 25 C unless noted.

To sample the sediment in the microcosm, the entire contents were thoroughly mixed with a mechanical stirrer. The desired amount of sediment was then removed with a sterile wide tip pipet.

All dilution water blanks were made with artificial sea water diluted to the same salinity as the water in the microcosms being sampled. Incubation was carried out for 10 days at 25 C. The base medium was composed of 0.5% peptone and 0.1% yeast extract prepared with artificial sea water of the desired salinity and modified by the addition of appropriate materials. Solidified media were prepared by the addition of 2% agar (Difco). The conventional spread plate technique was employed with incubation under air for aerobic counts and incubation under an atmosphere of 95% N₂-5% CO₂ for anaerobic counts. The spot plate technique employed for determining starch-, gelatin-, casein-, lipid-, and chitin-utilizing populations was conducted as follows: Three 0.01 ml portions of each sample were spotted on plates of media. After incubation, the plates were developed, and the number of positive reactions out of the three possible were recorded at each dilution. Numbers of organisms were calculated using a standard three-tube MPN table.

The standard three-tube MPN procedure was employed for estimating the numbers of sulfate-reducers, organisms capable of producing H₂S from organic sulfur, nitrate-reducers and ammonifiers.

Tests on dehydrogenase activity were carried out as follows: Two g portions of sediment were weighed into glass centrifuge tubes. Fifty to 100 mg of CaCO₃ powder were added to each tube and, with the exception of the blank, all tubes received 0.5 ml of a 1% solution of triphenyl tetrazolium chloride (TTC) prepared in 15 ppt artificial sea water. Samples were mixed, placed in a vacuum desiccator, evacuated for 10-15 min, and incubated under vacuum at 30 C. At the end of 22 h the vacuum was released and 10 ml of methanol added immediately to each tube. Samples were mixed and centrifuged at low speed for 2-3 min. The supernatant then was filtered through a cotton plugged funnel into a 50 ml volumetric flask and the methanol extraction procedure repeated using 10 ml amounts of methanol until color could no longer be extracted from the sample. The filtrate was made to volume (50 ml) using methanol. The color intensity was measured at 485 nm on a B&L Spectronic 20 colorimeter using the blank as a reference. Results were calculated from a standard curve and expressed as μ g triphenyl tetrazolium formazan (TPF) per g of sediment.

Studies on the oxygen consumption by organisms in sediments were conducted using conventional Warburg manometric techniques with glucose as a substrate. Sediment samples were amended with 1000 ppm carbofuran and incubated. This mixture was subsampled after 5 and 12 days incubation and 2.5 g wet weight was used in each Warburg flask. The salinity was 15 ppt and temperature was 25 C.

For the glucose mineralization experiments, the procedure employed was a modification of Harrison *et al.*¹² The major change involved the amount of C-14 glucose added (0.1 μ Ci per bottle) and the incubation time (1 h).

Proteolytic activity studies were monitored in terms of the change in soluble protein concentration within a sediment slurry to which peptone had been added as the protein source.

Five g of sediment and ten ml of sea water (15 ppt) containing 10 mg of Bacto-peptone was placed in 60 ml prescription bottles and incubated with shaking for 22 h at 25 C. The slurry was centrifuged at high speed to separate the liquid from the cells and sediment. The supernatant was checked for protein content using the method of Lowry *et al.*¹³

It was found that carbofuran reacted with the reagents used in the protein determination producing an overestimation of the protein concentration. Blanks without added peptone were used to compensate for this error.

Bioaccumulation-

These studies were conducted with culture #280 (*Benecka*) using mineral salts medium (15 ppt) with static incubation at 30 C. Inoculum consisted of cells from a 24 h, 30 C nutrient broth-grown culture which had been centrifuged, washed three times in saline and resuspended in the same solution. The dead cell controls were prepared by heating a portion of the cell suspension for 15 min at 80 C. For the studies utilizing mirex, a saturated solution was obtained by stirring 2 g of mirex in 3 l of mineral salts medium for 3 days. This solution then was filtered through a 0.45 μ membrane filter and the clear filtrate was considered to be saturated with mirex (approximately 10^{-3} g/ml). For studies utilizing carbofuran, a 150 ppm concentration was obtained in the test flask by dissolving 40 mg of carbofuran in 1 ml of ethanol and adding 0.75 ml into 100 ml of mineral salts medium.

The tests were conducted as follows: A 5% inoculum of both living and dead cell suspensions was separately placed in flasks containing mineral salts medium with 1% glucose and the test compounds. The test flask along with several control flasks was incubated for 48 h at 30 C under static conditions. Cells then were removed from the respective media by centrifugation followed by filtration through 0.45 μ membrane filter. The clear filtrate was analyzed for the test compound to determine how much of the material either adsorbed to the cells or was bioaccumulated. Bioaccumulation is defined here as the difference between the amount of test material adsorbed to the dead cells and the amount of material found in the live cells. Dry weights were determined initially and after 48 h on the test flask containing live cells to determine the increase in cell mass.

Isolation of Mirex- and Carbofuran-Utilizing Microorganisms-

Throughout the course of this contract a considerable effort was put forth to isolate mirex- and carbofuran-utilizing microorganisms. In a number of cases encouraging results were obtained but in no instance was it proven that a particular culture could use either of these compounds.

In regard to carbofuran, it was not anticipated that problems would arise in the isolation of an organism capable of utilizing this compound. Most of the work was directed toward obtaining a culture that was capable of utilizing carbofuran either as a sole carbon and/or nitrogen source. In most of the work minimal media were employed and it is entirely possible that richer media containing vitamins, accessory growth factors, etc. might have yielded different results. While efforts to isolate a carbofuran-utilizing microorganism were unsuccessful, this fact is not interpreted to mean that such organisms do not exist, but different methodology, different inocula, etc. should be employed in future work.

Similarly, no mirex-utilizing microorganisms were obtained even though a considerable amount of effort was expended in the search; and, at times results seemed quite encouraging. Cultures suspected of being mirex-utilizers (demonstrating growth in the presence of mirex and hexane and the concurrent lack of growth in hexane alone) failed to give any concrete evidence of mirex utilization. No conclusive verification could be obtained using manometric techniques and in general, results

were erratic. There is some evidence to support the hypothesis that some of the growth that was observed and some of the results obtained through manometry were the action of the organism on either mirex or a compound formed from mirex. Once again, failure to obtain a mirex-utilizing organism does not prove the non-existence of an organism possessing this characteristic, but rather reflects only the fact that under the conditions and materials used in this investigation none was obtained.

SECTION V RESULTS

MIREX AND CARBOFURAN

Adsorption Studies with Mirex

Water was saturated with mirex and the amount of mirex in the aqueous phase determined as a function of time by extraction with hydrocarbon solvent and analysis made by electron capture gas chromatography. The saturated solutions were made by stirring mirex in distilled water for 25 h followed by removal of the excess mirex with a millipore filter (0.45 μ). Table 1 summarizes the results for montmorillonite clay and Table 2 for kaolinite clay.

Table 1. ADSORPTION OF MIREX ON MONTMORILLONITE CLAY

Time days	Concentration mirex $\mu\text{g/ml}$				
	0 $\mu\text{g/ml}$ clay	0.9 $\mu\text{g/ml}$ clay	0.45 $\mu\text{g/ml}$ clay	0.09 $\mu\text{g/ml}$ clay	0.045 $\mu\text{g/ml}$ clay
4	0.003	0.004	0.002	0.003	0.002
7	0.004	0.002	0.001	0.003	0.001
14	0.001	0.002	0.003	0.001	0.006
30	0.002	0.00007	< 0.00003	< 0.00003	< 0.00003

No detectable mirex residues were found in the samples containing only clay. Adsorption to the glass vessels was not a major problem, as shown by the lack of disappearance of mirex in the systems without added clay. The precision of the data is not high; it is typical of this type of low-level residue analysis. For the zero clay samples which should have had constant mirex levels, the percent standard deviation is 52. It is clear that adsorption or slow chemical reaction does occur on the clay surface, ultimately removing all the pesticide from the aqueous phase.

Table 2. ADSORPTION OF MIREX ON KAOLINITE CLAY

Time days	Concentration mirex $\mu\text{g/ml}$				
	0 $\mu\text{g/ml}$ clay	0.9 $\mu\text{g/ml}$ clay	0.45 $\mu\text{g/ml}$ clay	0.09 $\mu\text{g/ml}$ clay	0.045 $\mu\text{g/ml}$ clay
4	0.003	0.002	0.002	0.003	0.002
7	0.004	0.0007	0.0008	0.0008	0.0008
14	0.001	0.0008	0.0007	0.0005	0.0004
30	0.002	0.00008	0.0001	< 0.00003	< 0.00003

Adsorption Studies with Carbofuran

Two independent studies were made on carbofuran adsorption to clay. In the first, montmorillonite clay was employed and the analyses conducted by ultraviolet adsorption at 276 nm. The apparent adsorption was about 10-15% after 35 days (Table 3). In the second study a solution of 189

Table 3. ADSORPTION STUDIES OF CARBOFURAN ON MONTMORILLONITE CLAY

Time days	Absorbances			
	0 ug/ml clay	0.06 ug/ml clay	0.13 ug/ml clay	0.6 ug/ml clay
1	1	--	0.84	--
2	0.93	--	0.87	--
3	--	0.85	0.84	0.87
7	1+	0.93	0.84	0.87
14	1+	0.92	0.86	0.91
21	1+	0.96	0.89	1+
28	1+	0.98	0.82	0.83
35	1+	0.95	0.83	0.83

ppm carbofuran in 0.05 M sodium acetate-acetic acid buffer at pH 5 was treated with acidified kaolinite clay such that the clay concentration was 1200 mg/l (Table 4).

Table 4. CARBOFURAN ADSORPTION ON KAOLINITE CLAY

Time	% Carbofuran remaining in solution
0	100
36 min	72
81 min	70
180 min	86
240 min	82
9 h - 46 min	68
21 h	68
28 h - 26 min	75
54 h	79

Apparently about 25% of the carbofuran is very rapidly adsorbed under this set of conditions, and then the system is at equilibrium and no further adsorption is observed.

Fate Studies with Mirex

The analytical method employed in this study had a coefficient of variation of about 25%. Table 5 lists the data obtained on disappearance of mirex from an estuarine sediment - water system. To this system 0.1 ppm mirex was introduced. A blank control consistently had undetectable levels of mirex.

The numbers listed for mirex are all the result of averaging three replicates. The recovery by this method is 70-80%, and thus the data show that no significant decrease in mirex concentration occurred during the duration of the experiment. No indication of the presence of dehalogenated derivatives was found. The samples were held in gallon jars during the experiment and had normal exposure to sunlight.

Table 5. FATE STUDIES - MIREX

Spiked 6-10-74 with 0.01 ppm Mirex	
Time/days	% Mirex recovered
0	75
1	79
2	88
4	83
8	76
16	79
31	78
40	71
53	68
63	77
70	79
81	72
88	71
100	70
113	62
123	68
130	64

Fate Studies with Carbofuran

The analyses were conducted by the heptafluorobutyric anhydride derivitization procedure described in the method section. The coefficient of variation for this method is about 50%. Recoveries were very poor when preliminary hydrolysis to free conjugated residues were employed and so the reported data are for free carbofurans. No detectable residues of 3-hydroxy- or 3-ketocarbofuran were found in any of these samples. The phenols would not be detected by this method. Table 6 is a compila-

Table 6. FATE STUDIES - CARBOFURAN

Time/days	% Carbofuran
0	81
1/2	50
1	86
2	53
4	61
6	55
10	64
13	54
16	67
41	33
49	24.7

tion of the data for disappearance of carbofuran from an estuarine sediment-water system. The initial concentration of carbofuran was 10 ppm. The data are more variable than for mirex. Occasionally one of the replicates showed no detectable carbofuran. These results were sometimes obtained on identical samples run in the same set on the same day. The reason for this erratic behavior has not been determined.

Hydrolysis and Aqueous Photochemical Studies with Mirex

Hydrolysis of mirex was studied in four systems: Distilled water, saline water 15 ppt, distilled water with 1 ppm arginine added, and distilled water with 1 ppm arginine added and exposed to sunlight. Table 7 summarizes the results.

Table 7. HYDROLYSIS OF MIREX

Time Days	Concentrations of mirex in $\mu\text{g/l}$			
	Distilled H_2O	15 ppt Salinity	1 $\mu\text{g/l}$ Arginine	1 $\mu\text{g/l}$ Arginine plus light
0	0.23	0.20	0.13	0.23
30	0.33	0.14	0.12	0.40
60	0.13	0.06	0.38	0.13
90	0.11	0.03	0.05	0.04

The numbers in Table 7 are for the most part the average of three replicates. The percent standard deviation for these data is about 65. While the precision is not high, the data show a definite trend towards disappearance of mirex with time.

Hydrolysis of Carbofuran

The hydrolysis of carbofuran was studied in 0.005 M sodium carbonate, buffered solution at 0 and 15 ppt, 25 C and pH 10. The initial concentration was about 300 ppm in all cases. Solutions were prepared by adding the appropriate amount of carbofuran in 30 ml of ethanol to 220 ml of the buffer solution. Aliquots were removed at various time intervals for analysis by liquid chromatography. These data are shown in Tables 8 and 9.

Table 8. HYDROLYSIS OF CARBOFURAN

15 ppt salinity pH 10 0.005M Na_2CO_3	
Time (min)	% Carbofuran remaining
4	100
9	94
25	80
35	77
45	72
55	70
65	69
75	65
85	63
95	64
105	60

Table 9. HYDROLYSIS OF CARBOFURAN

0 ppt salinity pH 10	
Time (min)	% Carbofuran remaining
4	100
10	89
20	83
35	73
45	71
55	64
65	62
85	57
95	55
105	55
115	55

The rate constants were calculated for both pseudo-first and second order kinetics. The second order rate expression gives a slightly better fit, but it was discovered that the pH was dropping slightly during the latter part of the reaction. When the experiment was repeated using 0.05 M buffer an excellent fit for first order kinetics was obtained up through 70% reaction. The rate constants in 0 and 15 ppt solution were identical within experimental error. The rate constant was greater in 0.05 M than in 0.005 M buffer as would be expected if the pH was dropping in the 0.005 M buffer. The first order rate constant was 0.011 sec^{-1} . This is about the same as that observed by Metcalf *et al.*⁶ at pH 9 and 37 C. Our earlier experiments showed that Metcalf's measurements were not based upon disappearance of carbofuran or appearance of phenol, but apparently were based on an artifact, and our experiments indicate that the rate constants they obtained were not related to the rate of hydrolysis of carbofuran.

Disc Assay Studies

The disc assay technique was employed as a rapid screening method to test the inhibitory effect of the mirex on a variety of pure cultures of bacteria isolated from an estuary. The first series of tests with the mirex produced zones of inhibition on 18, 2, 1 and 7 of the 20 cultures tested using four separate batches of mirex. The results were extremely erratic with three of the batches of mirex in that they did not consistently and/or repeatedly produce zones of inhibition.

Ten new batches of mirex were obtained from Allied Chemical Company and tested by means of "disc assay" against 20 bacterial cultures. No zones of inhibition were noted even when the experiment was replicated 5 times. Because of the results cited above, and the fact that gas chromatographic analyses indicated varying degrees of purity for the different batches, laboratory purified mirex was employed in subsequent tests unless otherwise noted.

Carbofuran failed to inhibit any of the 20 bacterial cultures when tested using the disc assay technique.

In another study using technical mirex and technical carbofuran, no inhibition was observed when tested against 596 isolates from estuarine and non-estuarine origin.

Effect of Mirex and Carbofuran on Growth Curves of Selected Cultures

Tests to determine the effect of mirex and carbofuran on pure cultures in liquid media (15 ppt salinity) were performed using 8 cultures and concentrations of the test compounds of 0 ppm, 0.1 ppm and 10 ppm. Essentially no reduction in counts occurred after shake incubation for 72 h (Tables 10 and 11). It should be pointed out that these tests were conducted using technical grade mirex rather than purified mirex.

In another series of tests optical density was employed to monitor growth. Mirex and carbofuran at concentrations of 10 and 1000 ppm failed to alter the growth response of any of the cultures tested: two species each of *Bacillus* (#222 and #275), *Achromobacter* (#223 and #274), and *Vibrio* (#221 and #295), and one species each of *Benecka* (#242), *Pseudomonas* (#297), and *Acinetobacter* (#173).

Results of Qualitative Studies on Effect of Mirex and Carbofuran

Qualitative tests using mirex and carbofuran in concentrations of 0.1, 10.0, and 100 ppm failed to give any evidence of inhibition of growth, glucose fermentation, or H_2S production by any of the cultures tested (7 glucose fermentors and 4 H_2S producers). Visual observations on solid media indicated no inhibition of growth or hydrolyzing ability of 16 gelatin-, 7 casein-, 1 lipid-, 6 starch- and 4 chitin-hydrolyzers.

Table 10. EFFECT OF VARYING CONCENTRATIONS OF MIREX ON GROWTH OF PURE CULTURES OF BACTERIA IN BROTH MEDIUM.

Culture	Mirex (technical)				
	0.0 ppm	0.1 ppm	1.0 ppm	10.0 ppm	
	Bacterial counts after 72 h incubation				
0h	No/ml	No/ml	No/ml	No/ml	No/ml
<i>Bacillus</i> (#222)	8.0×10^5	2.0×10^9	4.1×10^9	1.2×10^9	2.4×10^9
<i>Achromobacter</i> (#274)	1.6×10^5	1.5×10^9	9.9×10^8	7.8×10^8	1.0×10^7
<i>Vibrio</i> (#276)	5.1×10^5	4.2×10^8	1.4×10^9	7.8×10^8	9.5×10^8
<i>Benecke</i> (#242)	3.3×10^5	2.0×10^9	7.9×10^8	9.1×10^8	6.2×10^8
<i>Pseudomonas</i> (#297)	1.2×10^5	6.4×10^8	1.2×10^9	1.3×10^9	2.9×10^8
<i>Achromobacter</i> (#223)	2.0×10^5	3.3×10^8	4.8×10^8	1.6×10^8	0.6×10^8
<i>Enterobacter</i> (#189)	2.7×10^4	1.1×10^8	0.5×10^8	2.3×10^8	1.6×10^8
<i>Acinetobacter</i> (#173)	1.3×10^5	7.3×10^8	4.4×10^8	4.8×10^8	3.1×10^5

Conditions of test: Growth medium was nutrient broth prepared with 15 ppt salinity seawater; temp of incubation was 25 C. Plate counts were determined in duplicate.

Table 11. EFFECT OF VARYING CONCENTRATIONS OF CARBOFURAN ON GROWTH OF PURE CULTURES OF BACTERIA IN BROTH MEDIUM.

Culture	Carbofuran (technical)				
	0.0 ppm	0.1 ppm	1.0 ppm	10.0 ppm	
	Bacterial counts after 72 h incubation				
0h	No/ml	No/ml	No/ml	No/ml	No/ml
<i>Bacillus</i> (#222)	8.0×10^5	2.0×10^9	2.3×10^9	1.0×10^9	2.3×10^9
<i>Achromobacter</i> (#274)	1.6×10^5	1.5×10^9	7.1×10^8	8.8×10^8	8.7×10^8
<i>Vibrio</i> (#276)	5.1×10^5	4.2×10^8	2.3×10^8	---	8.5×10^8
<i>Benecke</i> (#242)	3.3×10^5	2.0×10^9	5.1×10^8	1.1×10^9	3.2×10^8
<i>Pseudomonas</i> (#297)	1.2×10^5	6.4×10^8	4.2×10^8	9.4×10^8	8.2×10^8
<i>Achromobacter</i> (#223)	2.0×10^5	3.3×10^8	2.6×10^8	4.7×10^8	4.8×10^8
<i>Enterobacter</i> (#189)	2.7×10^4	1.1×10^8	0.8×10^8	0.8×10^8	1.2×10^8
<i>Acinetobacter</i> (#173)	1.3×10^5	7.3×10^8	8.5×10^8	2.8×10^8	5.6×10^8

Conditions of tests: Growth medium was nutrient broth prepared with 15 ppt salinity seawater; temp of incubation was 25 C. Plate counts were determined in duplicate.

Effect of Mirex and Carbofuran on Methane Utilization

No inhibition by mirex or carbofuran of methane utilization by estuarine sediments was observed as determined by the total volume of gas consumed (Table 12). Although not of estuarine origin, a pure culture of *M. methanooxidans* was employed in a similar study (Table 13). In this case, however, all concentrations (0.1, 1.0, 10, and 100 ppm mirex and carbofuran) tested retarded gas consumption for several days, but after 10 days of incubation, the gas consumption in the presence of test compounds was the same as that in the absence of test compounds.

Table 12. EFFECT OF MIREX AND CARBOFURAN ON METHANE CONSUMPTION BY MICROFLORA IN ESTUARINE SEDIMENTS.

Test system	Time in Days										
	3	4	5	6	7	8	9	10	11	12	
Control	7 ^a	9	18	29	37	48	59	70	73	73	
Mirex 0.1 ppm	18	26	40	48	57	64	73	79	77	77	
Mirex 1.0 ppm	15	24	35	44	51	59	66	73	79	77	
Mirex 10.0 ppm	35	24	37	48	59	68	75	77	77	77	
Mirex 100.0 ppm	20	29	37	48	55	66	77	77	77	77	
Carbofuran 0.1 ppm	11	11	20	26	33	42	53	66	68	73	
Carbofuran 1.0 ppm	15	22	31	44	46	64	68	75	77	75	
Carbofuran 10.0 ppm	11	15	24	37	44	55	66	73	75	77	
Carbofuran 100.0 ppm	11	11	22	33	42	53	66	73	77	77	

^a ml of gas consumed

Conditions of test: Sohngen unit apparatus, medium 15 ppt salinity with mineral salts, incubated under gas atmosphere of 65% CH₄, 30% O₂ and 5% CO₂, static conditions. All values are the average of three determinations.

Table 13. EFFECT OF MIREX AND CARBOFURAN ON METHANE CONSUMPTION BY METHANOMONAS METHANOXIDANS.

Test system	Time in days									
	2	3	4	5	6	7	10	12	14	
Control	4 ^a	15	29	46	66	70	73	75	81	
Mirex 0.1 ppm	4	11	17	24	33	44	70	73	77	
Mirex 1.0 ppm	4	13	20	29	42	53	68	75	75	
Mirex 10.0 ppm	4	15	22	249	42	48	64	75	77	
Mirex 100.0 ppm	7	13	18	24	33	42	70	73	77	
Carbofuran 0.1 ppm	4	18	29	44	62	70	73	75	80	
Carbofuran 1.0 ppm	4	15	22	31	46	59	75	77	80	
Carbofuran 10.0 ppm	7	15	26	37	57	62	70	75	81	
Carbofuran 100.0 ppm	4	15	24	35	53	64	68	70	77	

^a ml of gas consumed

Condition of test: Sohngen unit apparatus, medium - mineral salts, incubated under gas atmosphere of 65% CH₄, 30% O₂ and 5% CO₂, static conditions. All values are the average of three determinations.

Effect of Mirex and Carbofuran on Ammonification

The data in Table 14 demonstrate that mirex and carbofuran (100 ppm) did not inhibit the production of ammonia from protein by four cultures of bacteria [*Benecka* (#280), *Pseudomonas* (#297), and two species of *Achromobacter* (#223 and #274)]. In the case of *Achromobacter* #274, substrate (protein) disappearance was decreased by the mirex even though the amount of ammonia produced was not decreased.

Table 14. EFFECT OF MIREX AND CARBOFURAN ON AMMONIFICATION BY FOUR PURE CULTURES OF BACTERIA.

Test	Culture No.	Optical density of culture				NH ₃ -N (mg/l)				Protein-N ^a (mg/l)	
		Days incubation				Days of incubation				initial	final
		1	2	3	4	1	2	3	4		
Blank	-	0.00	0.00	0.00	0.00	0	0	0	0	1125	1125
Control	223	0.06	0.12	0.28	0.35	0	0	35	37	1120	643
100 ppm Mirex	"	0.06	0.13	0.20	0.31	0	6.5	20	30	1125	648
100 ppm Carbofuran	"	0.06	0.12	0.27	0.32	0	0	22	28	1120	650
Control	274	0.26	0.32	0.50	0.62	10	13	23	61	1120	275
100 ppm Mirex	"	0.25	0.34	0.47	0.56	10	10	37	557	1110	553
100 ppm Carbofuran	"	0.23	0.32	0.42	0.53	10	10	40	70	1130	325
Control	297	0.11	0.20	0.25	0.38	10	24	63	58	1120	558
100 ppm Mirex	"	0.10	0.11	0.26	0.35	10	18	49	68	1110	555
100 ppm Carbofuran	"	0.11	0.12	0.24	0.36	10	18	45	56	1120	565
Control	280	0.60	0.95	1.10	1.15	18	77	140	125	1120	593
100 ppm Mirex	"	0.59	0.89	1.00	1.10	14	79	139	133	1130	583
100 ppm Carbofuran	"	0.62	0.95	1.10	1.20	10	78	140	150	1130	583

^a ppm protein-N of medium after filtration through 0.45 μ membrane filter. Conditions of test: medium: 15 ppt Kila salts + peptone. Incubation: 30 C, static conditions. All values are the average of two determinations.

Effect of Mirex and Carbofuran on Nitrate Reduction

The effect of mirex and carbofuran on nitrate reduction by *Achromobacter* #274 showed that nitrate utilization and nitrite production were essentially unaffected by concentrations of test compounds up to 100 ppm after 18 h of growth (Table 15). The data do suggest, however, that increasing concentrations of mirex and carbofuran delay the onset of nitrate reduction.

Table 15. EFFECT OF MIREX AND CARBOFURAN ON NITRATE-REDUCTION BY *ACHROMOBACTER* (#274).

Test system	Hours of incubation											
	6		12		18		24		36		48	
	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)
Control	101	-	81	0.75	70	1.48	66	1.66	60	1.75	54	1.75
Mirex 1 ppm	110	-	92	0.60	66	1.70	63	1.75	60	1.90	62	1.75
Mirex 10 ppm	120	-	94	1.0	66	1.50	65	1.65	63	1.85	68	1.80
Mirex 100 ppm	130	-	140	-	80	1.15	60	1.58	63	1.80	68	1.80
Carbofuran 1 ppm	132	-	137	-	72	1.30	66	1.50	70	1.70	71	1.60
Carbofuran 10 ppm	128	-	100	0.77	68	1.58	65	1.58	65	1.80	70	1.75
Carbofuran 100 ppm	124	-	137	-	106	0.50	68	0.75	66	1.65	81	1.50

All values are the average of two determinations.

Effect of Mirex and Carbofuran on Nitrification

To determine if mirex and carbofuran would affect nitrification, estuarine sediments were employed rather than pure cultures. Within the limits of the tests employed, no deleterious effect on the process was observed in terms of substrate disappearance (ammonia) or product formation (nitrite and nitrate) even after 20 days of incubation (Table 16).

Table 16. EFFECT OF MIREX AND CARBOFURAN ON NITRIFICATION BY MICROORGANISMS IN ESTUARINE SEDIMENTS.

Test system	Days of incubation											
	0			5			10			15		
	NH ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NH ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NH ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NH ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)
Blank	125	0	0	110	0	0	110	0	0	113	0	0
Control	128	0	0	105	0	0	105	trace	trace	100	trace	trace
Mirex												
1 ppm	125	0	0	93	0	0	91	"	"	83	"	<.025
10 ppm	120	0	0	102	0	0	100	"	"	100	"	<.025
100 ppm	119	0	0	105	0	0	105	"	"	100	"	<.025
Carbofuran												
1 ppm	120	0	0	96	0	0	91	"	"	88	"	<.025
10 ppm	118	0	0	103	0	0	106	"	"	100	"	<.025
100 ppm	120	0	0	105	0	0	100	"	"	100	"	<.025

Conditions of test: Medium: 15 ppt Rile salts + NH₄Cl + mineral salts, pH 7.0. Incubation: 30 C, shake conditions. All values are the average of two determinations.

Effect of Mirex and Carbofuran on Primary Productivity

As shown in Table 17, neither mirex nor carbofuran inhibited the metabolic activity of estuarine pond water, but did inhibit primary productivity. These experiments were repeated employing pond water enriched with phytoplankton. The results (Table 18) confirmed the lack of inhibition of metabolic activity by both compounds, confirmed the inhibition of primary productivity by carbofuran, but differed in the fact that mirex failed to inhibit primary productivity.

Table 17. EFFECT OF MIREX AND CARBOFURAN ON THE METABOLIC ACTIVITY AND PRIMARY PRODUCTIVITY OF ESTUARINE POND WATER.

Test system	Metabolic activity (mg O ₂ consumed/l in 12 h)	Primary productivity (mg O ₂ produced /l in 12 h)
Control	0.95	2.35
Acetone control	0.95	2.10
HgCl ₂ control	0.00	0.00
Mirex (1 ppm)	1.00	1.30
(10 ppm)	1.05	1.70
Carbofuran (1 ppm)	1.20	1.35
(10 ppm)	1.05	1.15

All values are the average of two determinations.

Table 18. EFFECT OF MIREX AND CARBOFURAN ON THE METABOLIC ACTIVITY AND PRIMARY PRODUCTIVITY OF ESTUARINE POND WATER ENRICHED FOR PHYTOPLANKTON.

Test system	Metabolic activity (mg O ₂ consumed/l in 12 h)	Primary productivity (mg O ₂ produced/l in 12 h)
Control	1.40	4.65
Acetone control	1.30	4.85
HgCl ₂ control	0.00	0.00
Mirex 1 ppm	1.30	5.00
10 ppm	1.10	5.00
Carbofuran 1 ppm	1.25	4.05
10 ppm	1.30	3.05

All values are the average of two determinations.

Effect of Mirex and Carbofuran on the Chemotactic Response of an Estuarine Bacterium

Preliminary experiments indicated that concentrations of 10 and 50 ppm of mirex and carbofuran were not toxic to this bacterium while a concentration of 250 ppm was definitely lethal. Additionally, it was demonstrated that no significant increase in bacterial numbers occurred during an incubation period of 54 min. With asparagine as the attractant, a positive chemotactic response toward this compound occurred with increasing concentrations from 10^{-6} M to 10^{-2} M. The bacterium failed to demonstrate a chemotactic response in tests employing 10^{-6} M asparagine in the presence of 10 ppm mirex or carbofuran (Table 19), but gave a positive chemotactic response in the presence of 10^{-6} M

Table 19. COMPARISON OF CHEMOTACTIC RESPONSE OF THE ESTUARINE BACTERIUM TOWARD 10^{-6} M ASPARAGINE IN THE PRESENCE OR ABSENCE OF 10 PPM MIREX OR CARBOFURAN.

Sample No.	1	2	3	4
Contents in vial	Cells in Rila	Cells in Rila	Cells in Rila	Cells in Rila
Contents in capillary tube	Acetone residue ----- Rila -----	Acetone residue ----- Rila ----- 10^{-6} M Asparagine	Acetone residue ----- Mirex residue ----- Rila ----- 10^{-6} M Asparagine	Acetone residue ----- Carbofuran residue ----- Rila ----- 10^{-6} M Asparagine
(a)	30 34 Ave. 36 35 39	40 50 Ave. 50 50 58	51 62 Ave. 71 65 76	56 66 Ave. 67 70 90
No. colonies on plate at 10^{-3} dilution	51 57 Ave. 66 60 68	79 82 Ave. 96 89 99	86 109 Ave. 130 114 133	84 118 Ave. 118 112 128
(a)	30 30 Ave. 50 41 53	56 66 Ave. 66 66 76	74 83 Ave. 84 82 87	72 87 Ave. 87 86 98

(a) Replicates of separate experiments.

asparagine and 50 ppm mirex or carbofuran (Table 20). This positive chemotactic response was nullified when 10^{-4} M asparagine was employed along with 50 ppm mirex or carbofuran (Table 21). In tests employing nutrient broth (0.08%) as the attractant, no chemotactic response was observed in the presence of 10 ppm mirex or 10 or 50 ppm carbofuran (Table 22 & 23). A positive chemotactic response was observed with nutrient broth (0.08%) in the presence of 50 ppm mirex. This positive response to 50 ppm mirex could be neutralized by incubating the mirex and nutrient broth in combination for a period of 3.5 h.

Microcosm Studies with Mirex and Carbofuran

The systems, termed here as microcosms, were not true microcosms or mini-ecosystems in that they were static with no input of light energy or detrital organic matter which is constantly fed into the natural sediment ecosystem. During construction of the microcosms, the sediments were disturbed which undoubtedly altered their nature and possibly the niche of some of the microorganisms present in the system. Even with these drawbacks it was felt that static microcosm studies were important in establishing the effect of pesticides upon the mixed microbial flora within the sediments.

Effect on Bacterial Populations-

In microcosm studies the numbers of aerobic heterotrophic bacteria, anaerobic heterotrophic bacteria and nine biochemical groups of microorganisms were followed for a period of four weeks after the microcosm sediments were amended with various concentrations of mirex or carbofuran. The results are summarized in Tables 24 through 27 (mirex) and Tables 28 through 31 (carbofuran). Changes in the microbial populations at the 0.1 ppm level could not necessarily be linked to the pesticide because acetone was added as a diluent.

Table 20. COMPARISON OF CHEMOTACTIC RESPONSE OF THE ESTUARINE BACTERIUM TOWARD 10^{-6} M ASPARAGINE IN THE PRESENCE OR ABSENCE OF 50 PPM MIREX OR CARBOFURAN.

Sample No.	1		2		3		4	
Contents in vial	Cells in Rila		Cells in Rila		Cells in Rila		Cells in Rila	
	Acetone residue		Acetone residue		Acetone residue		Acetone residue	
Contents in capillary tube	-----		-----		Mirex residue		Carbofuran residue	
	Rila		Rila		Rila		Rila	
	-----		10^{-6} M Asparagine		10^{-6} M Asparagine		10^{-6} M Asparagine	
No. colonies	11		21		63		47	
on plate	14	Ave.	23	Ave.	74	Ave.	54	Ave.
at 10^{-4}	16	15	33	28	79	76	58	55
dilution	20		34		86		60	

Table 21. COMPARISON OF CHEMOTACTIC RESPONSE OF THE ESTUARINE BACTERIUM TOWARD 10^{-4} M ASPARAGINE IN THE PRESENCE OR ABSENCE OF 50 PPM MIREX OR CARBOFURAN.

Sample No.	1		2		3		4	
Contents in vial	Cells in Rila		Cells in Rila		Cells in Rila		Cells in Rila	
Contents in capillary tube	Acetone residue		Acetone residue		Acetone residue		Acetone residue	
	-----		-----		Mirex residue		Carbofuran residue	
	Rila		Rila		Rila		Rila	
	-----		10^{-4} M Asparagine		10^{-4} M Asparagine		10^{-4} M Asparagine	
No. colonies on plate at 10^{-3} dilution	36		49		53		80	
	(a) 41	Ave.	58	Ave.	62	Ave.	93	Ave.
	44	42	65	60	73	66	98	93
	45		66		74		102	
(b)	36		65		--		80	
	43	Ave.	65	Ave.	--		84	Ave.
	48	47	70	70	--	--	90	88
	60		81		--		96	
(a)	11		57		76		56	
	14	Ave.	62	Ave.	85	Ave.	67	Ave.
	15	16	77	69	86	83	70	68
	22		79		86		79	
Overall ave.	35		66		74		83	

(a) Replicates obtained from experiment in which time sequence began with sample no. 1 followed sequentially by samples no. 2, 3, and 4.

(b) Replicates obtained from experiment in which time sequence began with sample no. 4 followed sequentially by samples no. 3, 2, and 1.

Table 22. COMPARISON OF CHEMOTACTIC RESPONSE OF THE ESTUARINE BACTERIUM TOWARD 10 PPM MIREX OR CARBOFURAN IN THE PRESENCE OF 0.06% NUTRIENT BROTH.

Sample No.	1		2		3		4	
Contents in vial	Cells in Rila		Cells in Rila		Cells in Rila		Cells in Rila	
Contents in capillary tube	Acetone residue		Acetone residue		Acetone residue		Acetone residue	
	-----		-----		Mirex residue		Carbofuran residue	
	Rila		Rila		Rila		Rila	
	-----		Nutrient broth		Nutrient broth		Nutrient broth	
No. colonies on plate at 10^{-3} dilution	69		107		140		96	
	72	Ave.	125	Ave.	153	Ave.	110	Ave.
	73	72	131	124	160	156	111	109
	74		131		173		118	

Table 23. COMPARISON OF CHEMOTACTIC RESPONSE OF THE ESTUARINE BACTERIUM TOWARD 50 PPM MIREX OR CARBOFU-
RAN (AS RESIDUE) IN THE PRESENCE OF 0.08% NUTRIENT BROTH.

Sample No.	1		2		3		4	
Contents in vial	Cells in Rila		Cells in Rila		Cells in Rila		Cells in Rila	
Contents in capillary tube	Acetone residue		Acetone residue		Acetone residue		Acetone residue	
	-----		-----		Mirex residue		Carbofuran residue	
	Rila		Rila		Rila		Rila	
	-----		Nutrient broth		Nutrient broth		Nutrient broth	
(a)	34		48		131		97	
	34	Ave.	60	Ave.	141	Ave.	97	Ave.
	35	35	73	65	143	140	106	104
	38		80		144		116	
(b)	29		95		225		134	
	32	Ave.	107	Ave.	247	Ave.	136	Ave.
	38	36	119	110	251	244	167	153
	43		121		255		175	
(a)	26		93		249		118	
	34	Ave.	105	Ave.	283	Ave.	156	Ave.
	43	36	115	111	307	289	156	149
	43		132		316		161	
Overall ave.	36		95		224		135	

(a) Replicates obtained from experiment in which time sequence began with sample no. 1 followed sequentially by samples no. 2, 3, and 4.

(b) Replicates obtained from experiment in which time sequence began with sample no. 4 followed sequentially by samples no. 3, 2, and 1.

Table 24. MICROBIAL COUNTS ON SEDIMENT SAMPLES FROM MICROCOSH TANKS IMMEDIATELY AFTER AMENDING WITH MIREX.

Microbial group	#/ml	Control		Amended with mirex							
				0.1 ppm		10 ppm		1000 ppm		10,000 ppm	
		Tank 11	Tank 16	Tank 12	Tank 17	Tank 13	Tank 18	Tank 14	Tank 19	Tank 15	Tank 20
Nitrate reducers	10 ⁵	4.3 ^a	9.3	4.3	9.3	2.4	2.9	2.1	4.3	1.5	43.0
		2.4 ^a	2.4	9.3	46.0	2.5	9.3	2.4	2.4	0.93	15.0
Ammonifiers	10 ⁵	4.3	2.4	12.0	9.3	9.3	2.4	2.4	2.4	2.4	2.4
		1.5	9.3	2.4	7.5	2.4	2.4	2.4	4.3	4.3	9.3
H ₂ S producers	10 ⁴	2.3	2.3	15.0	43.0	4.3	9.3	15.0	4.3	4.3	9.3
		4.3	3.9	2.3	15.0	9.3	4.3	4.3	24.0	2.3	b
Sulfate reducers	10 ³	24.0	43.0	4.3	15.0	24.0	15.0	4.3	15.0	1.5	9.3
		9.3	4.3	2.3	24.0	9.3	15.0	9.3	4.3	24.0	b
Lipid hydrolyzers	10 ⁵	2.4	1.5	1.2	4.3	1.5	0.93	4.3	2.4	7.5	2.4
		2.4	2.4	2.4	2.4	9.3	4.3	1.5	4.3	1.5	2.4
Casein hydrolyzers	10 ⁵	9.3	4.3	2.4	7.5	9.3	1.2	4.3	2.4	2.4	15.0
		4.3	2.4	9.3	21.0	24.0	7.5	2.4	2.3	0.93	4.3
Gelatin hydrolyzers	10 ⁵	4.3	24.0	4.3	15.0	2.4	4.3	4.3	4.3	4.3	9.3
		2.4	24.0	24.0	9.3	0.23	2.4	9.3	4.3	7.5	4.3
Starch hydrolyzers	10 ⁵	2.4	2.4	0.43	9.3	2.4	2.4	9.3	3.9	2.4	2.4
		9.3	7.5	2.4	24.0	460.0	4.3	2.4	4.3	21.0	0.93
Chitin hydrolyzers	10 ⁴	9.4	2.3	4.3	4.3	4.3	9.3	4.3	4.3	9.3	0.9
		2.3	4.3	2.3	12.0	9.3	2.9	0.93	9.3	75.0	2.3
Aerobic plate count	10 ⁶	1.2	1.6	1.6	1.6	1.4	1.0	1.4	1.6	1.1	1.6
		1.1	1.1	1.1	1.3	1.3	1.0	0.9	1.6	1.3	1.4
Anaerobic plate count		b	b	b	b	b	b	b	b	b	b

^a Replicate counts

^b Missing value

Table 25. MICROBIAL COUNTS ON SEDIMENT SAMPLES FROM MICROCOSM TANKS ONE WEEK AFTER AMENDING WITH MIREX.

Microbial group	#/ml x	Amended with mirex									
		Control		0.1 ppm		10 ppm		1000 ppm		10,000 ppm	
		Tank 11	Tank 16	Tank 12	Tank 17	Tank 13	Tank 18	Tank 14	Tank 19	Tank 15	Tank 20
Nitrate reducers	10 ⁵	9.3 ^a 9.3 ^a	9.3 21.0	150.0 93.0	43.0 240.0	2.1 15.0	24.0 75.0	43.0 4.3	24.0 4.3	4.3 9.3	43.0 24.0
Ammonifiers	10 ⁵	2.4 43.0	7.5 9.3	9.3 23.0	24.0 93.0	7.5 2.4	4.3 4.3	93.0 7.5	4.3 4.3	9.3 4.3	24.0 9.3
H ₂ S producers	10 ⁴	4.3 4.3	43.0 9.3	2.3 4.3	9.3 9.3	2.3 9.3	4.3 2.4	2.3 9.3	2.3 2.3	4.3 15.0	7.5 4.3
Sulfate reducers	10 ⁴	2.4 2.4	2.4 4.3	7.5 4.3	0.93 2.4	2.4 9.3	2.4 2.4	2.3 0.43	2.4 4.3	4.3 1.5	9.3 0.39
Lipid hydrolyzers	10 ⁵	4.3 2.4	43.0 7.5	9.3 240.0	9.3 24.0	9.3 9.3	4.3 9.3	4.3 4.3	7.5 4.3	9.3 9.3	4.3 9.3
Casein hydrolyzers	10 ⁵	2.4 2.4	4.3 9.3	9.3 9.3	4.3 24.0	24.0 4.3	2.4 0.93	2.4 2.4	2.4 2.1	9.3 7.5	9.3 4.3
Gelatin hydrolyzers	10 ⁵	4.3 9.3	9.3 15.0	24.0 43.0	93.0 240.0	9.3 9.3	4.3 46.0	4.3 4.4	2.5 21.0	4.3 7.5	4.3 7.5
Starch hydrolyzers	10 ⁵	2.4 4.3	1.5 2.4	2.4 0.93	4.3 4.3	4.3 1.5	0.9 2.4	4.3 2.4	2.4 4.3	9.3 2.4	2.4 2.4
Chitin hydrolyzers	10 ⁴	2.4 7.5	2.4 2.3	2.4 9.3	0.04 2.3	1.5 0.9	3.9 2.4	2.3 1.1	0.9 1.5	4.3 2.3	2.3 1.5
Aerobic plate count	10 ⁶	3.7 3.9	9.6 9.5	49.0 35.0	18.1 19.6	5.1 6.1	4.2 4.4	4.4 4.1	5.7 4.3	5.2 4.3	6.9 6.6
Anaerobic plate count	10 ⁵	7.0 10.0	15.0 17.0	79.0 95.0	113.0 138.0	11.0 11.0	7.0 11.0	9.0 8.0	8.0 10.0	7.0 7.0	15.0 15.0

^a Replicate counts

Table 26. MICROBIAL COUNTS ON SEDIMENT SAMPLES FROM MICROCOSM TANKS TWO WEEKS AFTER AMENDING WITH MIREX.

Microbial group	#/ml x	Amended with mirex									
		Control		0.1 ppm		10 ppm		1000 ppm		10,000 ppm	
		Tank 11	Tank 16	Tank 12	Tank 17	Tank 13	Tank 18	Tank 14	Tank 19	Tank 15	Tank 20
Nitrate reducers	10 ⁵	240.0 ^a 210.0 ^a	43.0 15.0	93.0 93.0	150.0 240.0	15.0 15.0	24.0 4.3	93.0 15.0	24.0 9.3	39.0 4.3	24.0 43.0
Ammonifiers	10 ⁵	15.0 4.3	4.3 4.3	93.0 240.0	93.0 240.0	9.3 2.4	4.3 4.3	2.4 3.9	2.4 2.4	4.3 4.3	9.3 7.5
H ₂ S producers	10 ⁴	7.5 9.3	2.3 24.0	7.5 2.4	43.0 9.3	1.5 4.3	24.0 9.3	2.3 2.3	4.3 9.3	1.5 2.3	15.0 9.3
Sulfate reducers	10 ³	43.0 4.3	9.3 9.3	9.3 15.0	9.3 4.3	4.3 9.3	3.9 24.0	24.0 43.0	9.3 24.0	9.3 9.3	15.0 9.3
Lipid hydrolyzers	10 ⁵	9.3 4.3	4.3 9.3	24.0 24.0	93.0 93.0	24.0 24.0	4.3 4.3	2.4 2.4	9.3 9.3	43.0 43.0	9.3 9.3
Casein hydrolyzers	10 ⁵	9.3 24.0	b b	4.3 43.0	b b	4.3 2.4	b b	b b	b b	b b	9.3 9.3
Gelatin hydrolyzers	10 ⁵	9.3 9.3	15.0 21.0	24.0 43.0	93.0 24.0	24.0 24.0	4.3 9.3	12.0 4.3	9.3 9.3	9.3 43.0	b b
Starch hydrolyzers	10 ⁵	3.9 2.4	b b	2.4 2.3	b b	2.4 1.5	b b	2.4 b	b b	0.93 4.3	b 2.4
Chitin hydrolyzers	10 ⁴	2.3 0.9	0.4 1.1	0.9 0.36	b b	0.9 b	2.3 0.4	0.7 2.1	b b	0.9 2.3	4.3 0.4
Aerobic plate count	10 ⁶	18.1 16.1	19.5 20.8	41.0 30.0	46.0 38.0	3.9 5.2	5.3 5.4	8.5 4.2	4.3 5.1	4.1 4.6	10.7 9.9
Anaerobic plate count	10 ⁵	14.7 15.1	24.7 23.1	141.0 151.0	155.0 154.0	9.3 11.9	10.6 10.0	22.9 28.2	12.9 12.2	13.0 11.5	34.0 37.0

^a Replicate counts^b Missing value

Table 27. MICROBIAL COUNTS ON SEDIMENT SAMPLES FROM MICROCOSM TANKS FOUR WEEKS AFTER AMENDING WITH MIREX.

Microbial group	#/ml x	Amended with mirex									
		Control		0.1 ppm		10 ppm		1000 ppm		10,000 ppm	
		Tank 11	Tank 16	Tank 12	Tank 17	Tank 13	Tank 18	Tank 14	Tank 19	Tank 15	Tank 20
Nitrate reducers	10 ⁶	4.6 ^a 4.2 ^a	1.1 2.2	13.0 1.8	3.2 11.0	1.4 7.9	2.4 0.5	7.9 3.3	3.3 2.1	1.8 1.2	7.9 4.9
Ammonifiers	10 ⁶	1.1 1.7	0.8 2.8	7.9 4.9	4.7 7.0	1.3 3.3	1.4 3.3	3.3 2.4	0.5 3.3	1.1 3.3	0.8 4.9
H ₂ S producers	10 ⁶	1.7 1.3	2.8 0.8	4.9 1.3	4.9 1.1	3.3 0.8	3.3 0.5	2.2 0.7	4.9 1.3	2.4 1.4	2.4 0.8
Sulfate reducers	10 ⁴	1.7 4.9	0.3 79.0	0.7 17.0	0.8 17.0	0.8 3.3	0.2 24.0	0.5 3.3	0.8 33.0	0.8 7.9	0.5 24.0
Lipid hydrolyzers	10 ⁵	4.9 7.9	79.0 3.3	17.0 17.0	17.0 22.0	3.3 7.0	24.0 0.8	3.3 1.4	33.0 3.3	7.9 3.3	24.0 2.4
Casein hydrolyzers	10 ⁵	7.9 4.9	3.3 7.9	17.0 33.0	22.0 17.0	7.0 2.4	0.8 7.9	1.4 4.9	3.3 4.9	3.3 1.3	2.4 7.9
Gelatin hydrolyzers	10 ⁵	33.0 13.0	24.0 13.0	130.0 79.0	240.0 240.0	33.0 33.0	7.9 7.9	3.3 24.0	17.0 11.0	4.9 7.9	24.0 33.0
Starch hydrolyzers	10 ⁵	2.2 4.9	4.9 2.2	3.3 3.3	0.8 3.3	3.3 4.9	0.8 1.3	1.7 1.3	0.4 28.0	1.3 2.4	2.4 17.0
Chitin hydrolyzers	10 ⁴	0.4 19.8	0.2 25.4	2.3 47.0	1.4 62.0	0.2 15.0	0.8 8.3	1.2 8.7	0.5 9.5	0.8 7.1	1.7 11.2
Aerobic plate count	10 ⁶	18.4 20.3	23.0 101.0	57.0 150.0	59.0 158.0	16.7 50.0	6.9 34.0	7.6 34.0	7.8 31.0	8.7 8	13.4 50.0
Anaerobic plate count	10 ⁵	16.1	87.0	135.0	141.0	64.0	34.0	32.0	24.0	36.0	57.0

^a Replicate counts^b Missing value

Table 28. MICROBIAL COUNTS ON SEDIMENT SAMPLES FROM MICROCOSM TANKS IMMEDIATELY AFTER AMENDING WITH CARBOFURAN.

Microbial group	#/ml x	Amended with carbofuran									
		Control		0.1 ppm		10 ppm		1000 ppm		10,000 ppm	
		Tank 21	Tank 26	Tank 27	Tank 28	Tank 29	Tank 30	Tank 31	Tank 32	Tank 33	Tank 34
Nitrate reducers	10 ⁵	3.3	4.9	0.8	2.1	2.1	1.7	1.4	0.8	1.3	2.4
Ammonifiers	10 ⁵	7.9	3.3	1.3	4.9	7.0	1.7	7.0	1.1	1.7	11.0
H ₂ S producers	10 ⁵	1.3	0.3	1.3	0.3	0.2	0.3	0.5	0.5	1.1	0.5
Sulfate reducers	10 ⁵	1.3	0.7	7.9	2.4	0.8	1.4	1.7	0.7	3.3	0.5
Lipid hydrolyzers	10 ⁵	4.9	4.9	4.9	2.4	4.9	4.9	2.4	4.9	3.3	1.3
Casein hydrolyzers	10 ⁵	1.9	2.4	3.3	2.4	3.3	2.1	2.4	2.4	3.3	1.7
Gelatin hydrolyzers	10 ⁵	0.8	1.9	24.0	2.2	3.3	4.9	2.4	13.0	7.9	2.8
Starch hydrolyzers	10 ⁵	0.8	4.9	3.3	1.7	1.4	2.4	2.4	2.4	1.4	1.3
Chitin hydrolyzers	10 ⁵	2.7	0.7	4.3	2.3	2.3	0.9	17.0	4.9	1.3	3.4
Aerobic plate count	10 ⁵	1.3	7.6	8.4	5.6	5.2	6.6	5.9	6.4	7.7	8.7
Anaerobic plate count	10 ⁵	2.9	3.4	2.6	2.4	2.8	2.9	3.7	3.5	2.7	3.4

Table 29. MICROBIAL COUNTS ON SEDIMENT SAMPLES FROM MICROCOSM TANKS ONE WEEK AFTER AMENDING WITH CARBOPURAN.

Microbial group	#/ml x	Amended with carbopuran									
		Control		0.1 ppm		10 ppm		1000 ppm		10,000 ppm	
		Tank 25	Tank 26	Tank 27	Tank 28	Tank 29	Tank 30	Tank 31	Tank 32	Tank 33	Tank 34
Nitrate reducers	10 ⁵	49.0	1.7	79.0	79.0	240.0	49.0	7.0	13.0	2.8	11.0
Ammonifiers	10 ⁵	7.9	4.9	13.0	3.3	7.9	14.0	33.0	34.0	33.0	14.0
H ₂ S producers	10 ⁵	0.5	2.4	1.3	1.7	7.9	1.4	0.03	0.3	3.3	0.5
Sulfate reducers	10 ⁴	3.3	3.3	2.2	2.8	2.4	2.8	4.9	3.3	0.95	0.8
Lipid hydrolyzers	10 ⁵	3.3	11.0	22.0	7.9	7.9	4.9	49.0	7.9	33.0	33.0
Casein hydrolyzers	10 ⁵	4.9	2.4	3.3	3.3	2.2	1.3	11.0	13.0	49.0	13.0
Gelatin hydrolyzers	10 ⁵	4.9	7.9	17.0	7.9	13.0	4.9	2.4	49.0	24.0	2.4
Starch hydrolyzers	10 ⁵	2.2	2.2	4.9	4.9	0.8	1.3	4.9	2.2	7.9	7.9
Chitin hydrolyzers	10 ⁴	4.9	4.6	3.3	2.2	2.7	2.2	1.4	0.8	0.8	0.5
Aerobic plate count	10 ⁶	10.4	11.2	12.6	11.7	6.7	8.5	3.8	4.7	5.9	6.1
Anaerobic plate count	10 ⁶	6.6	6.2	10.0	9.2	4.6	5.4	3.3	3.8	5.8	4.1

Table 30. MICROBIAL COUNTS ON SEDIMENT SAMPLES FROM MICROCOSM TANKS TWO WEEKS AFTER AMENDING WITH CARBOPURAN.

Microbial group	#/ml x	Amended with carbopuran									
		Control		0.1 ppm		10 ppm		1000 ppm		10,000 ppm	
		Tank 25	Tank 26	Tank 27	Tank 28	Tank 29	Tank 30	Tank 31	Tank 32	Tank 33	Tank 34
Nitrate reducers	10 ⁵	79.0	49.0	4.9	170.0	4.5	21.0	79.0	240.0	130.0	240.0
Ammonifiers	10 ⁵	1.3	4.9	7.9	13.0	4.9	14.0	46.0	13.0	17.0	0.8
H ₂ S producers	10 ⁵	.08	.08	0.3	0.5	1.3	0.5	0.5	0.8	1.3	1.7
Sulfate reducers	10 ⁴	3.3	7.9	3.3	2.4	3.3	4.9	1.3	1.3	5.8	0.2
Lipid hydrolyzers	10 ⁵	3.3	13.0	4.9	33.0	22.0	7.9	79.0	33.0	79.0	33.0
Casein hydrolyzers	10 ⁵	4.3	3.1	4.6	7.9	7.0	4.9	17.0	13.0	17.0	17.0
Gelatin hydrolyzers	10 ⁵	3.3	7.9	2.4	13.0	4.9	11.0	130.0	22.0	240.0	130.0
Starch hydrolyzers	10 ⁵	1.3	1.2	2.2	4.9	1.3	1.3	1.8	1.1	4.9	3.3
Chitin hydrolyzers	10 ⁴	0.9	3.3	0.5	4.9	3.3	4.9	0.8	0	0	0.4
Aerobic plate count	10 ⁶	14.0	19.2	22.4	19.5	20.5	14.5	21.9	26.3	35.0	32.0
Anaerobic plate count	10 ⁶	7.8	10.8	11.0	12.4	10.8	9.8	12.5	14.6	15.1	13.2

Table 31. MICROBIAL COUNTS ON SEDIMENT SAMPLES FROM MICROCOSM TANKS FOUR WEEKS AFTER AMENDING WITH CARBOFURAN.

Microbial Group	#/±1 x	Control		Amended with carbofuran							
				0.1 ppm		10 ppm		1000 ppm		10,000 ppm	
		Tank 25	Tank 26	Tank 27	Tank 28	Tank 29	Tank 30	Tank 31	Tank 32	Tank 33	Tank 34
Nitrate reducers	10 ⁶	7.9	7.0	2.6	0.4	0.3	4.0	24.0	17.0	24.0	28.0
Ammonifiers	10 ⁵	1.7	13.0	4.9	4.9	3.3	3.3	33.0	140.0	170.0	170.0
H ₂ S Producers	10 ⁵	0.3	0.3	1.3	0.7	0.3	3.3	1.1	1.1	0.24	1.3
Sulfate reducers	10 ⁴	1.3	2.8	0.8	1.3	0.3	1.3	0.8	0.8	0.22	0.5
Lipid hydrolyzers	10 ⁵	2.4	22.0	3.3	79.0	22.0	2.4	24.0	79.0	33.0	33.0
Casein hydrolyzers	10 ⁵	4.6	3.3	9.5	18.0	3.5	7.0	33.0	33.0	49.0	49.0
Gelatin hydrolyzers	10 ⁵	7.9	33.0	4.9	7.9	24.0	33.0	240.0	240.0	49.0	22.0
Starch hydrolyzers	10 ⁵	1.7	2.1	3.3	1.1	1.3	2.4	2.4	1.1	1.7	7.9
Chitin hydrolyzers	10 ⁴	1.1	1.3	1.1	1.7	2.6	1.3	0	0.7	0.6	0.2
Aerobic Plate count	10 ⁶	13.1	16.7	20.7	17.2	15.5	17.5	34.0	39.0	23.4	27.0
Anaerobic Plate count	10 ⁶	8.9	13.3	19.6	18.1	13.7	7.3	13.1	20.0	18.2	18.4

In the studies with mirex, it was noted that concentrations in excess of 10 ppm caused a slight but significant reduction in the two and four week aerobic plate counts as compared to the control. Morphological characteristics of the colonies on plates from all treated samples were similar to those on plates from the control samples. Anaerobic plate counts were not significantly different from the control at any concentration of mirex. Statistical analysis of the data gathered on the other nine biochemical groups of organisms was conducted without showing any difference between treatments and controls.

After two weeks incubation, the samples amended with 1000 ppm and 10,000 ppm carbofuran showed an increase in the number of aerobic bacteria, anaerobic bacteria and some of the other groups of heterotrophic bacteria over numbers found in control sediments. Colonies on the plates from both treated and control samples were morphologically the same. At a concentration of 10 ppm, populations in treated and control samples were about the same.

Since the aerobic heterotrophic bacterial plate counts were easy to perform and could be replicated with a minimum of effort to produce a direct number rather than a statistically derived one as produced by the MPN counts, it was decided to use the plate count as the measure of effect of the pesticide treatments in further studies.

Salinity-Temperature Studies-

The effect of various salinities and incubation temperatures on the bacterial population of the sediment was measured in the presence and absence of both mirex and carbofuran in a concentration of 100 ppm. The temperatures and salinities employed were approximately the maximum and minimum values recorded in the estuary.

Overall, the bacterial populations in mirex-amended sediments did not differ from the controls at any of the salinities and temperatures tested. Statistical analysis of the data verified this observation (Table 32). The previous study showed that mirex suppressed the bacterial populations. However, this difference was attributed to lot-to-lot variability in the mirex since in the previous study a different lot of mirex was employed.

The addition of carbofuran to samples produced significantly higher bacterial counts as compared to the control under all conditions of salinity and temperature tested (Table 33). None of these temperature-salinity conditions produced results noticeably different from the other.

Table 32. MEAN BACTERIAL COUNTS ($\times 10^4$) IN SEDIMENTS AMENDED WITH 100 PPM MIREX AND HELD UNDER VARIOUS CONDITIONS OF SALINITY AND TEMPERATURE.

Incubation period after pesticide added (days)	15 ppt - 10 C		15 ppt - 21 C		15 ppt - 30 C		5 ppt - 21 C		30 ppt - 21 C	
	Control	Mirex	Control	Mirex	Control	Mirex	Control	Mirex	Control	Mirex
0	113 ^a	143 ^c	91	98	96	168 ^c	54	54	111	110
4	442	420	662	651	722	772	645	648	532	487
9	743	697	942	843	933	878	1066	938	807	695
16	802	792	892	845	1155	907 ^b	798	755	748	682
22	868	824	1177	1046	[3885] [10,136] ^c		710	668	966	952
30	680	679	980	1136	608	645	745	769	1080	846 ^b
44	972	926	[8300]	4485	918	802	1236	977	[5638]	1370 ^c

^a - Mean of three replicates with five plates counted on each replicate

[] - Poor agreement among replicates which differed by one log in count.

^b - Treatment counts significantly different from control counts at 95% level as determined using t-test.

^c - Treatment counts significantly different from control counts at 99% level as determined using t-test.

Dehydrogenase Activity-

Dehydrogenase activity is used as an indication of anaerobic metabolism in sediments. Pamatmat and Bhagwat¹⁴ pointed out that anaerobic metabolism may greatly exceed aerobic metabolism in sediments. Therefore, a measure of this activity was used to evaluate the effect of pesticides on microbial activity.

Since previous studies had indicated variability among lots of technical grade pesticides, purified preparations of the pesticides as well as the technical grade material were employed in these studies.

As shown in Table 34, preparations of mirex added to sediments produced no alteration in the dehydrogenase activity when compared to untreated sediments. At some incubation times, the dehydrogenase activity was increased slightly and in others it was decreased slightly when compared to the control. Differences in levels of mirex did not alter this phenomenon.

All preparations of carbofuran at a concentration of 100 ppm significantly depressed the dehydrogenase activity (Table 35). At concentrations of 10 and 1 ppm carbofuran, depression of dehydrogenase activity was noted in only a few cases and in some cases, the activity was higher in amended sediments than in untreated ones. Overall, there appeared to be little difference among the different preparations of carbofuran.

Table 33. MEAN BACTERIAL COUNTS ($\times 10^4$) IN SEDIMENTS AMENDED WITH 100 PPM CARBOFURAN AND HELD UNDER VARIOUS CONDITIONS OF SALINITY AND TEMPERATURE.

Incubation period after pesticide added (days)	15 ppt - 10 C		15 ppt - 21 C		15 ppt - 30 C		5 ppt - 21 C		30 ppt - 21 C	
	Control	Carbofuran	Control	Carbofuran	Control	Carbofuran	Control	Carbofuran	Control	Carbofuran
0	113 ^a	167 ^c	91	123 ^c	96	147 ^c	54	55	111	105
4	462	638 ^c	662	636	722	1075 ^c	645	589	532	530
9	743	880 ^b	942	1009	933	1416 ^c	1066	1227 ^b	807	660
16	802	1344 ^c	892	1300 ^c	1155	1487 ^b	798	1359 ^c	748	2053 ^c
22	868	1463 ^c	1177	[5086] ^c	[3885]	1438 ^c	710	2116 ^c	966	1392 ^c
30	680	1335 ^c	980	1326 ^c	608	1242 ^c	745	1498 ^c	1080	1374 ^c
44	972	1601 ^c	[8300]	2013 ^b	918	620 ^c	1236	1932 ^c	[5638]	[10275] ^b

- ^a - Mean of three replicates with five plates counted on each replicate.
^b - Poor agreement among replicates which differed by one log in count.
^c - Treatment counts significantly different from control counts at 95% level as determined using t-test.
^c - Treatment counts significantly different from control counts at 99% level as determined using t-test.

Table 34. DEHYDROGENASE ACTIVITY IN SEDIMENTS TREATED WITH DIFFERENT PREPARATIONS AND CONCENTRATIONS OF MIREX.

Addition to sediment	Incubation period after pesticide added				
	Initial	7 days	14 days	21 days	28 days
-----1000ppm-----					
Untreated	22 ^a	180	190	171	127
Technical mirex	20 (0.1) ^b	182 (NS)	174 (NS)	155 (NS)	106 (.05)
Purified mirex	22 (NS)	174 (NS)	196 (NS)	169 (NS)	139 (NS)
-----100 ppm-----					
Untreated	23	164	137	212	172
Technical mirex	24 (NS)	165 (NS)	211 (.001)	191 (NS)	181 (NS)
Purified mirex	25 (NS)	207 (.001)	244 (.001)	231 (NS)	213 (.001)
-----10 ppm-----					
Untreated	21	108	98	115	88
Technical mirex	23 (NS)	119 (NS)	105 (NS)	54 (.001)	102 (.01)
Purified mirex	24 (.10)	158 (.01)	127 (.01)	139 (.05)	134 (.001)
-----1 ppm-----					
Untreated	31	152	158	150	104
Technical mirex	43 (.001)	181 (.01)	162 (NS)	151 (NS)	158 (.001)
Purified mirex	34 (NS)	157 (NS)	198 (.10)	148 (NS)	158 (.001)

- ^a Mean dehydrogenase activity (log of TPF produced per gram of wet sediment) from six replicates.
^b Significance level of difference between treated and untreated samples as determined by the t-test.
 NS means not significant at the 0.1 level.

Table 35. DEHYDROGENASE ACTIVITY IN SEDIMENTS TREATED WITH DIFFERENT PREPARATIONS AND CONCENTRATION OF CARBOFURAN.

Addition to sediment	Incubation period after pesticide added			
	Initial	6 days	11 days	20 days
Amended with 100 ppm				
Untreated	113 ^a	89	57	62
Technical carbofuran	86 (.05) b	23 (.001)	16 (.001)	19 (.001)
Purified carbofuran	105 (NS)	22 (.001)	25 (.001)	16 (.001)
Water washed carbofuran	124 (NS)	40 (.001)	24 (.001)	29 (.001)
Amended with 10 ppm				
Untreated	49	36	37	34
Technical carbofuran	68 (.01)	38 (NS)	45 (.01)	42 (.10)
Purified carbofuran	47 (NS)	36 (NS)	41 (NS)	21 (.001)
Water washed carbofuran	71 (.001)	37 (NS)	40 (NS)	59 (.001)
Amended with 1 ppm				
Untreated	115	73	57	24
Technical carbofuran	121 (NS)	62 (.05)	43 (.001)	32 (.001)
Purified carbofuran	140 (.001)	77 (NS)	53 (NS)	32 (.01)
Water washed carbofuran	148 (.001)	46 (.001)	41 (.001)	35 (.001)

^a Mean dehydrogenase activity (μ g of TPF produced per gram of wet sediments) from six replicates.

^b Significance level of difference between treated and untreated samples as determined by the t-test. NS means not significant at the 0.1 level.

The question arose as to why the dehydrogenase activity in carbofuran amended sediments decreased, while the bacterial numbers increased. To answer this question, seven pure cultures of bacteria of the types which predominated in sediments were isolated and inoculated into autoclave-sterilized sediment which had been enriched with 1% glucose. As a positive control, enriched sterile sediment was inoculated with fresh bay sediment. In all inoculated sediments, the bacteria increased in number, but only in the sediments inoculated with the fresh sediment did the dehydrogenase activity increase. This indicated that these aerobic heterotrophic bacteria did not significantly contribute to the dehydrogenase activity within the sediments, and their increase in numbers would not be expected to increase dehydrogenase activity.

Proteolytic Activity-

Protein utilization studies were conducted to determine if the pesticides affected the assimilatory power of the sediment microflora. As shown in Table 36 neither of the pesticides at any of the concentrations tested appeared to greatly change the amount of protein utilized.

Glucose Mineralization-

Radioisotopic studies were employed to determine the effect of various concentrations of mirex and carbofuran on the carbon dioxide released during the mineralization of glucose. Results of these studies are shown in Table 37.

The addition of carbofuran in concentrations of 1000 and 100 ppm significantly depressed the rate at which glucose was converted into carbon dioxide. After four weeks incubation with carbofuran

Table 36. PROTEOLYTIC ACTIVITY OF MICROORGANISMS IN SEDIMENT SAMPLES AMENDED WITH CARBOFURAN AND MIREX.

Concentration of pesticide added to sediment (ppm)	Sediment incubation period after pesticides added				
	Initial	1 week	2 weeks	3 weeks	4 weeks
----- Mirex amended sediments -----					
0	81.0 ^a	85.0	75.6	82.0	76.0
1000	79.0	82.0	77.0	75.0	74.0
100	80.0	86.0	77.1	74.0	72.0
10	79.0	87.0	80.6	78.0	74.0
1	73.0	87.5	83.7	76.0	75.0
----- Carbofuran amended sediments -----					
0	81.7	90.3	82.7	74.7	82.0
100	---	71.0	81.3	69.9	80.3
10	75.2	72.6	83.7	69.9	83.5
1	80.8	77.1	81.3	70.6	80.0

^a - Percent of added peptone protein utilized during 22 hour incubation period.
Average of four replicates.

Table 37. MINERALIZATION OF GLUCOSE BY MICROORGANISMS IN SEDIMENTS AMENDED WITH VARIOUS CONCENTRATIONS OF CARBOFURAN AND MIREX.

Concentration of pesticide added to sediment (ppm)	Sediment incubation period after pesticide added				
	Initial	1 week	2 weeks	3 weeks	4 weeks
----- Mirex amended sediments -----					
0	1158 ^a	1715	1328	941	1040
1000	922 ^c -21.0 ^b	2225 ^c +29.7	1277 - 3.8	877 - 6.8	890 -14.4
100	1033 ^c -10.8	1690 - 1.5	1317 - 0.8	829 -11.9	1123 + 7.9
10	915 ^c -21.0	1818 + 5.9	1246 - 6.2	855 - 9.1	910 -12.6
1	897 ^c -22.5	1583 - 7.7	1369 + 3.1	891 - 5.3	894 -14.0
----- Carbofuran amended sediments -----					
0	518	1165	1022	719	1758
1000	225 ^c -56.6	233 ^c -80.3	230 ^c -77.5	169 ^c -76.5	219 ^c -87.5
100	375 ^c -27.6	904 ^c -22.4	765 ^c -25.3	554 ^c -22.9	1244 ^c -29.2
10	295 ^c -43.1	1149 - 1.4	987 - 3.4	721 + 0.3	1789 + 1.8
1	281 ^c -45.8	1261 + 8.2	901 - 3.0	603 ^c -16.1	1728 - 1.7

^a - Level of radioactivity (CPM) in the CO₂ produced from the mineralization of C¹⁴ - glucose. Average of four replicates.

^b - Percent difference between control and treated sediments.

^c - Significantly different from control (P>0.05). t-test.

at the above concentration, carbon dioxide production was reduced by 87.5% and 29.2%, respectively. No significant alteration in the carbon dioxide evolution rate was detected at lower concentrations of carbofuran or at any concentration of mirex tested.

Respirometric Studies-

With the rate of glucose mineralization being decreased in sediments amended with carbofuran, it was expected that the oxygen consumption during glucose utilization may also be affected. As shown in Table 38, the amount of oxygen consumed during the breakdown of glucose was reduced in amended sediments. This reduction was comparable percentagewise to the reduction in glucose mineralization.

Table 38. OXYGEN CONSUMPTION DURING GLUCOSE UTILIZATION BY ORGANISMS IN SEDIMENTS AMENDED WITH 1000 PPM CARBOFURAN.

	Total oxygen consumption (μ l)	Oxygen consumption corrected for endogenous respiration (μ l)	Reduction in oxygen consumption of amended sediment (%)
Five days after sediments amended -----			
Untreated sediment (endogenous respiration)	224*	100	100
Untreated sediment with glucose	303	79	21
Amended sediment (endogenous respiration)	225	100	100
Amended sediment with glucose	236	11	89.1
Twelve days after sediments amended -----			
Untreated sediment (endogenous respiration)	193	100	100
Untreated sediment with glucose	221	28	72
Amended sediment (endogenous respiration)	175	100	100
Amended sediment with glucose	188	12	87.1

* Average of two replicate samples over a six hour period.

Hydrogen Sulfide Production-

Microcosms prepared with beach sand were amended with 1000 ppm mirex, 1000 ppm carbofuran or 100 ppm carbofuran, and were incubated in the dark. After seven days, the control microcosm and the one amended with mirex developed a dark gray-colored reducing layer below the sand water interface. This layer was absent in the carbofuran amended samples. Subsequent studies showed a concentration greater than 10 ppm carbofuran was required to produce this effect.

Hydrogen sulfide, which causes the blackening effect, is produced either from organic sulfur compounds or from sulfate reduction. Previous microcosm studies have shown that the addition of carbofuran does not reduce the number of organisms capable of making these transformations in sediments. Thus it is speculated that carbofuran in some way prevents the production of hydrogen sulfide without killing the organisms.

Bioaccumulation Studies on Mirex and Carbofuran-

Table 39 lists the results of a 48 h study on the bioaccumulation of carbofuran by culture #280. Cells in the test system were removed by centrifugation and the carbofuran in the liquid was analyzed by liquid chromatography. Peak heights were compared to an internal standard (carbaryl) in each sample and the carbofuran to internal standard ratio was established for each test. Relative values were obtained by making sample #2 equal to unity since it should not have a loss of carbofuran. The relative values found in Table 39 are not significantly different with respect to tests with live cells, dead cells and test with no cells. Sample #7, which contained the live cells + 1% glucose, had the lowest relative value, but it also has a 27% increase in total solids due to increase in cell mass. These data indicate that the carbofuran was removed from the test system by adsorption to the cells and not by bioaccumulation.

Table 39. BIOACCUMULATION STUDIES USING CARBOFURAN.

Sample #	Test system	Ratio carbofuran/internal standard	Relative value (#2 = 1)
Controls (no cells)			
#1	100 ml Mineral salts	-	-
#2	100 ml Mineral salts + 150 ppm carbofuran	1.22	1.0
#3	100 ml Mineral salts + 1% glucose	-	-
#4	100 ml Mineral salts + 150 ppm carbofuran + 1% glucose	1.14	0.97
Live cells			
#5	100 ml Mineral salts + 5% cells	-	-
#6	100 ml Mineral salts + 5% cells + 150 ppm carbofuran	1.14	0.93
#7	100 ml Mineral salts + 5% cells + 150 ppm carbofuran + 1% glucose	1.09	0.89
Dead cells			
#8	100 ml Mineral salts + 5% cells	-	-
#9	100 ml Mineral salts + 5% cells + 150 ppm carbofuran	1.12	0.92
#10	100 ml Mineral salts + 5% cells + 150 ppm carbofuran + 1% glucose	1.15	0.94

- = No carbofuran detected, all values are the average of two determinations.

The protocol for the bioaccumulation studies with mirex was the same as that for the studies with carbofuran. After 48 h incubation, the cells were removed by centrifugation and the mirex remaining in the liquid was determined by standard techniques outlined by the official method of the Food and Drug Administration (Anonymous¹⁵) using electron capture gas liquid chromatography. The results are listed in Table 40. By considering the amount of mirex found in the liquid of samples #7 & 8 to be 100%, it can be noted that the amount adsorbed by the dead cells (samples #9 & 10) was about 95% of the total present. The live cells (samples #11 & 12) adsorbed only 88% of the mirex even though there was a 27% increase in total mass of cells due to multiplication. Within the limitations of the test system used, it is concluded that there was no bioaccumulation of mirex occurring using culture #280.

Table 40. BIOACCUMULATION STUDIES OF MIREX BY CULTURE #280 (BENEFICA).

Sample #	Test system	Total mirex in liquid (ug)	Percent remaining in liquid
1	200 ml Mineral salts	-	-
2	200 ml Mineral salts	-	-
3	200 ml Mineral salts + 1% glucose	-	-
4	200 ml Mineral salts + 1% glucose	-	-
5	200 ml Mineral salts + 5% cells	-	-
6	200 ml Mineral salts + 5% cells	-	-
7	200 ml Mineral salts + mirex + 1% glucose	20.14	100
8	200 ml Mineral salts + mirex + 1% glucose	22.22	100
9	200 ml Mineral salts + 5% dead cells + mirex + 1% glucose	0.94	4
10	200 ml Mineral salts + 5% dead cells + mirex + 1% glucose	1.31	6
11	200 ml Mineral salts + 5% live cells + mirex + 1% glucose	2.71	12
12	200 ml Mineral salts + 5% live cells + mirex + 1% glucose	2.71	12

DERIVATIVES OF MIREX AND CARBOFURAN

As reported previously, the inhibitory effect of mirex (technical) varied from batch to batch and was erratic even when multiple tests were conducted using the same batch. Purified mirex samples never exerted an inhibitory effect when tested using the disc assay technique. There was reason to believe that contamination arises in aged mirex samples since older samples of mirex were toxic to the greatest number of bacterial cultures.

Since hexachlorocyclopentadiene is the starting material for mirex synthesis, this compound is a potential contaminant in the mirex. However, disc assay tests failed to indicate any inhibition with the 20 bacterial cultures tested when it was added to mirex at a concentration of 1:1000.

Chemical analyses of technical grade mirex demonstrated the presence of hexachlorobenzene (HCB) in some of the mirex samples. However, this compound was not found to be toxic to any of the 20 bacterial cultures as determined by disc assay tests.

Since Alley *et al.*¹ have identified 10-hydrogen; 5,10-dihydrogen; 8-hydrogen; and 2,8-dihydrogen derivatives of mirex as photodegradation products, and Ivie, Dorough, and Alley¹⁶ reported kepone to be a degradation product of mirex, these compounds along with reduced kepone were tested against a number of bacterial cultures using the disc assay technique. Neither the 8-hydrogen derivative of mirex nor the 2,8-dihydrogen derivative of mirex inhibited any of the 10 cultures tested. The 10-hydrogen derivative of mirex and the 5,10-dihydrogen derivative of mirex inhibited all 5 of the five cultures tested, while the kepone and reduced kepone both inhibited 10 of the 20 cultures tested. It is interesting to note that neither of the two products with substitutions on the 2 and 8 positions of the mirex molecule were inhibitory, while the four products with substitutions on the 5 and/or 10 positions of the mirex molecule were highly toxic to the test microorganisms. Whether these compounds were actual contaminants in the mirex employed in this investigation is unknown.

All attempts to isolate the specific compound(s) responsible for the inhibitory activity of the technical mirex failed.

In another series of experiments water-moistened mirex alone and in combination with HCB were exposed to UV irradiation (20 min under high pressure mercury lamp, 1800-6000 Å) and employed in disc assay tests. The data (Table 41) clearly illustrate that irradiation of the mirex resulted in a material which was toxic to *Achromobacter* #274. Curiously, irradiated mirex plus HCB appeared to have a greater toxicity than irradiated mirex alone, even though nonirradiated mirex plus HCB and irradiated HCB were nontoxic. Unfortunately, no record is available on the storage conditions under which the batches of mirex were kept, and it could well be that they had a history of exposure to moisture and UV irradiation over a period of time.

Table 41. RESULTS OF "DISC ASSAYS" OF UV IRRADIATED HEXACHLOROBENZENE AND MIREX AGAINST CULTURE #274 (*ACHROMOBACTER*).

Test system	Zone of inhibition	
	UV treatment	No UV treatment
Purified mirex	+	-
Technical mirex	++	-
HCB	-	-
HCB + purified mirex (2:100)	+++	-
HCB + purified mirex (2:10)	++++	-

* Increase in "+"s represents an increase in the diameter of the zone of inhibition

Since no organisms capable of utilizing either mirex or carbofuran were obtained, tests on the impact of metabolites were impossible. Under the circumstances, the above named photoproducts of mirex and 3-hydroxycarbofuran and 3-ketocarbofuran (known degradation products of carbofuran) were employed in subsequent tests.

Results of Qualitative Studies on Effect of Mirex and Carbofuran Derivatives

Qualitative studies on the effect of derivatives of mirex and carbofuran on the hydrolysis of starch, lipid, chitin, casein, and gelatin by several pure cultures of estuarine bacteria were conducted. Four cultures, #222, #276, #242 and #280, were tested for chitin hydrolysis. Only cultures #242 and #280 showed the ability to hydrolysis chitin and neither of these cultures was affected by any of the test compounds. Six cultures were utilized for the tests involving starch hydrolysis. Kepone and reduced kepone inhibited growth of three out of six while the remaining compounds showed no inhibition. None of the test compounds affected the growth of seven casein hydrolyzers. Growth of two out of six gelatin hydrolyzers was inhibited by kepone, and one out of six by reduced kepone. Only one culture, #242, illustrated the ability to hydrolyze lipid and it was not affected by any of the test compounds.

Effect of Mirex and Carbofuran Derivatives on Methane Consumption by Microflora in Estuarine Sediments

Table 42 shows the results of the effect of mirex and carbofuran derivatives on the process of methane consumption by the microflora in estuarine sediments after 12 days of incubation at 30 C under static conditions. No inhibition was noted with any of the test compounds; instead, there are indications of a stimulatory effect on the rate of methane consumption by several of the derivatives. Since the test system (sediments) is so complex in nature, it is difficult to properly assess this phenomenon. One possible explanation could be that these compounds are retarding certain groups of bacteria that might otherwise compete with the methane consumers.

Table 42. EFFECT OF MIREX AND CARBOFURAN DERIVATIVES ON METHANE CONSUMPTION BY MICROFLORA IN ESTUARINE SEDIMENTS.

Test system	Time in days											
	2	3	4	5	6	7	8	9	10	11	12	
Blank	0 ^a	0	0	0	0	2	4	4	4	4	4	
Control	4	9	11	18	24	31	35	42	53	64	75	
3-Hydroxycarbofuran-10 ppm	4	9	11	15	20	29	35	46	62	70	77	
1 ppm	4	9	11	15	11	19	22	44	55	64	73	
3-Ketocarbofuran-10 ppm	4	11	15	24	31	42	51	59	70	79	86	
1 ppm	4	13	18	26	37	48	56	75	81	84	86	
Kepon-10 ppm	2	9	11	18	22	26	35	44	53	64	75	
1 ppm	4	9	13	22	29	40	46	57	73	81	86	
Reduced Kepon-10 ppm	4	11	13	24	33	44	53	66	77	84	86	
1 ppm	7	11	18	26	35	46	55	68	81	86	86	
2,8-Dihydrogen mirex-10 ppm	4	11	13	22	29	40	48	59	73	79	84	
1 ppm	4	11	13	24	35	48	57	70	79	79	84	
10-Hydrogen 10 ppm	7	15	20	31	44	59	73	84	88	88	88	
and 5,10-dihydrogen-1 ppm mirex	4	9	13	20	29	40	46	59	73	81	86	

^a ml of gas consumed.

Conditions of test: Schöngen unit apparatus, medium 15 ppt salinity with mineral salts incubated under gas atmosphere of 65% CH₄, 30% O₂ and 5% CO₂, static conditions. All values are the average of three determinations.

Effect of Mirex and Carbofuran Derivatives on Ammonification

A pure culture, #280, was used to study the effect of mirex and carbofuran derivatives on the process of ammonification. After 3 days of incubation at 30 C under static conditions no difference was noted between the tests containing the derivatives and the control when monitoring substrate disappearance (protein-N) and product formation (NH₃-N). These results are illustrated in Table 43.

Effect of Mirex and Carbofuran Derivatives on Nitrate Reduction

The results of the effect of mirex and carbofuran derivatives on nitrate reduction are found in Table 44. Cultures containing the test compounds (concentration 1 and 10 ppm) showed the same rate of substrate utilization (NO₃-N) and product formation (NO₂-N) as did the control.

Table 43. EFFECT OF DERIVATIVES OF MIREX AND CARBOFURAN ON AMMONIFICATION BY A PURE CULTURE OF AN ESTUARINE BACTERIUM - #280 (BENECKA).

Test system	NH ₃ -N (mg/l)				Protein-N ^a (mg/l)	
	Initial	Day 1	Day 2	Day 3	Initial	Final
Blank	0	0	0	0	1200	1200
Control	0	119	195	270	1200	698
3-Hydroxycarbofuran 10 ppm	0	121	200	272	1200	710
1 ppm	0	125	230	310	1200	710
3-Ketocarbofuran 10 ppm	0	125	192	280	1200	727
1 ppm	0	125	227	292	1200	710
Kepone 10 ppm	0	75	100	240	1200	698
1 ppm	0	127	210	297	1200	715
Reduced kepone 10 ppm	0	120	200	282	1200	690
1 ppm	0	133	235	302	1200	715
2,8-Dihydrogen mirex 10 ppm	0	125	200	275	1200	698
1 ppm	0	127	220	300	1200	710
10-Hydrogen and 5,10-dihydrogen- mirex 10 ppm	0	123	202	277	1200	710
1 ppm	0	123	240	300	1200	727

^a Protein-N of medium after filtration through 0.45 μ membrane filter. Conditions of test: Medium: 15 ppt Hila sea salts, mineral salts. Incubation: 30 C, static conditions. All values are the average of two determinations.

Table 44. EFFECT OF MIREX DERIVATIVES AND CARBOFURAN DERIVATIVES ON NITRATE-REDUCTION BY AN ESTUARINE BACTERIUM - #280 (BENECKA).

Test System	Hours of Incubation							
	0		6		12		24	
	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NO ₂ -N (mg/l)
Blank	100	0	100	0	100	0	100	0
Control	100	0	48	0.7	7.5	2.4	<1.0	2.8
3-Ketocarbofuran 10 ppm	100	0	75	.65	14.0	2.4	<1.0	3.2
1 ppm	100	0	64	.63	12.0	2.4	<1.0	3.2
3-Hydroxycarbofuran 10 ppm	100	0	72	.64	5.0	2.1	<1.0	3.1
1 ppm	100	0	69	.75	7.5	2.1	<1.0	3.2
Kepone 10 ppm	100	0	88	.73	7.0	2.1	<1.0	3.0
1 ppm	100	0	90	.62	24.0	2.1	<1.0	3.2
Reduced kepone 10 ppm	100	0	84	.74	7.0	2.4	<1.0	3.1
1 ppm	100	0	78	.73	7.5	2.3	<1.0	3.2
2,8-Dihydrogen mirex 10 ppm	100	0	56	.77	10.0	2.3	<1.0	3.0
1 ppm	100	0	72	.70	7.5	2.1	<1.0	3.0
10-Hydrogen and 5,10-dihydrogen- mirex 10 ppm	100	0	93	.68	7.5	2.4	<1.0	3.0
1 ppm	100	0	88	.70	6.5	2.4	<1.0	3.0

All values are the average of two determinations.

Effect of Mirex and Carbofuran Derivatives on Nitrification

Three of the test compounds, 3-hydroxycarbofuran, kepone and reduced kepone at concentrations of 1 and 10 ppm retarded nitrification as illustrated in Table 45 by reduction in both disappearance of substrate (NH₃-N) and product formation (NO₂-N and NO₃-N). No effect on nitrification was found with 3-ketocarbofuran, 2,8-dihydrogen mirex and the mixture of 10-hydrogen and 5,10-dihydrogen mirex.

Table 45. EFFECT OF MIREX AND CARBOFURAN DERIVATIVES ON NITRIFICATION BY MICROORGANISMS IN ESTUARINE SEDIMENTS.

Test system	Days of incubation											
	0			5			10			15		
	NH ₄ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NH ₄ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NH ₄ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	NH ₄ -N (mg/l)	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)
Blank	130*	-	-	130	-	-	130	-	-	130	-	-
Control	130	-	-	128	-	-	125	trace	trace	100	.02	.6
3-Ketocarbofuran 10 ppm	130	-	-	128	-	-	124	+	+	100	.02	.4
1 ppm	130	-	-	130	-	-	117	+	+	90	.01	.4
3-Hydroxycarbofuran 10 ppm	130	-	-	130	-	-	127	+	+	105	+	<.1
1 ppm	130	-	-	128	-	-	115	+	+	90	+	<.1
Kepon 10 ppm	130	-	-	128	-	-	124	+	+	120	+	<.1
1 ppm	130	-	-	130	-	-	115	+	+	110	+	<.1
Reduced kepon 10 10 ppm	130	-	-	128	-	-	125	+	+	99	.01	<.1
1 ppm	130	-	-	130	-	-	120	+	+	90	.01	<.1
2,8-Dihydrogen mirex 10 ppm	130	-	-	130	-	-	123	+	+	105	.02	<.1
1 ppm	130	-	-	128	-	-	123	+	+	90	.02	<.1
10-Hydrogen and 5,10-dihydrogen mirex 10 ppm	130	-	-	130	-	-	120	+	+	103	.02	<.1
1 ppm	130	-	-	130	-	-	120	+	+	90	.02	<.1

Conditions of test: Medium: 15 ppt Rila salts + NH₄ 1 + mineral salts, pH 7.0, incubation: 10 C, shake conditions. All values are the average of two determinations.

Effect of Mirex and Carbofuran Derivatives on Metabolic Activity and Primary Productivity

The results of the effect of mirex derivatives and carbofuran derivatives on the primary productivity of natural estuarine waters are reported in Table 46. In light of the low primary productivity,

Table 46. THE EFFECT OF MIREX AND CARBOFURAN DERIVATIVES ON THE METABOLIC ACTIVITY AND PRIMARY PRODUCTIVITY OF ESTUARINE POND WATER.

Test System	Metabolic activity (mg O ₂ consumed/l in 12 h)	Primary productivity (mg O ₂ produced/l in 12 h)
Control	0.95	2.35
Acetone control	0.95	2.10
HgCl ₂ Control	0.00	0.00
Reduced kepon (1 ppm) (10 ppm)	0.90 0.85	0.80 0.50
10-hydrogen der. of mirex (1 ppm) (10 ppm)	1.25 1.10	1.80 1.50
8-hydrogen der. of mirex (1 ppm) (10 ppm)	1.00 1.15	1.50 1.70
2, 8-dihydrogen der. of mirex (2 ppm) (10 ppm)	1.15 1.05	1.65 1.85
3-ketocarbofuran (1 ppm) (10 ppm)	1.10 0.80	1.20 0.95
3-hydroxycarbofuran (1 ppm) (10 ppm)	1.20 1.10	1.60 1.35

All values are the average of two determinations.

this experiment was rerun using a water previously enriched in phytoplankton population. Table 47 lists the results of this experiment. No growth inhibition was observed with the 10-hydrogen mirex or 8-hydrogen mirex. Both concentrations (1 and 10 ppm) of the reduced kepone, kepone, 3-ketocarbafuran, 3-hydroxycarbafuran reduced the primary productivity. A reduction in the primary productivity also was noted with the 2,8-dihydrogen mirex, but the reduction was not as great as for the above compounds.

Effect of "Wing Derivatives" of Mirex (Mixture of 10-hydrogen and 5,10-dihydrogen mirex) on the Dehydrogenase Activity in Sediments

Table 48 lists the results of the effect of a mixture of "wing derivatives" of mirex (10-hydrogen and 5,10-dihydrogen mirex) on dehydrogenase activity in sediments. No reduction in dehydrogenase activity was observed with any of the concentrations of the test compounds. On the other hand, several of the concentrations showed a stimulatory effect on dehydrogenase activity of sediments sampled up to 28 days after application of the test compound.

Table 47. THE EFFECT OF MIREX AND CARBOFURAN DERIVATIVES ON THE METABOLIC ACTIVITY AND PRIMARY PRODUCTIVITY OF ESTUARINE POND WATER ENRICHED FOR PHYTOPLANKTON.

Test system	Metabolic activity (mg O ₂ consumed/l in 12 h)	Primary productivity (mg O ₂ produced /l in 12 h)
Control	1.40	4.65
Acetone control	1.30	4.85
HgCl ₂ control	0.00	0.00
Reduced kepone 1 ppm	1.25	1.15
10 ppm	1.00	0.30
Kepone 1 ppm	1.20	3.60
10 ppm	1.20	1.80
10-Hydrogen mirex 1 ppm	1.20	5.15
10 ppm	1.20	4.60
8-Hydrogen mirex 1 ppm	1.35	5.20
10 ppm	1.35	4.85
2,8-Dihydrogen mirex 1 ppm	1.35	4.40
10 ppm	1.40	4.05
3-Ketocarbafuran 1 ppm	1.45	4.10
10 ppm	1.45	3.05
3-Hydroxycarbafuran 1 ppm	1.40	3.90
10 ppm	1.10	2.70

All values are the average of two determinations.

Table 48. DEHYDROGENASE ACTIVITY IN SEDIMENTS TREATED WITH DERIVATIVES OF MIREX.

Addition to sediment	Period after pesticide added				
	Initial	7 days	14 days	21 days	28 days
-----1000 ppm-----					
Untreated	22 ^a	180	190	171	127
"wing derivatives" of mirex	19 (.05)	168 (NS)	184 (NS)	193 (NS)	166 (.01)
-----100 ppm-----					
Untreated	23	164	137	212	172
"wing derivatives" of mirex	24 (NS)	205 (.001)	222 (.001)	260 (.02)	224 (.001)
-----10 ppm-----					
Untreated	21	108	98	115	88
"wing derivatives" of mirex	19 (NS)	150 (.001)	129 (.001)	108 (NS)	144 (.001)
-----1 ppm-----					
Untreated	31	152	158	150	104
"wing derivatives" of mirex	32 (NS)	142 (NS)	205 (.01)	204 (.01)	176 (.001)

^a Mean dehydrogenase activity (μ g of TPF produced per gram of wet sediment) from six replicates.
NS means not significant at the 0.1 level.

SECTION VI

SUMMARY

At least 95% of the mirex in aqueous solution is removed by adsorption to organic matter (dead bacterial cells), kaolinite clay and montmorillonite clay within 2, 7 and 30 days, respectively. Only 8-15% of the carbofuran is removed from aqueous solution by adsorption to these materials.

No significant decrease in mirex concentration occurred in a sediment-water system exposed to sunlight for 130 days, but there was evidence of some disappearance in purely aqueous solutions. Contrarywise, approximately 75% of the carbofuran disappears within 49 days in sediment-water system, but no detectable residues of 3-hydroxy- or 3-ketocarbofuran were found.

In disc assay tests, carbofuran failed to inhibit the growth of any of the 20 bacterial isolates tested while the results with mirex varied from batch to batch with one batch producing zones of inhibition on 18 of the 20 cultures. Gas chromatographic analyses demonstrated that different batches of mirex contained varying amounts of unidentified impurities. For the most part, the remaining studies were conducted with purified mirex.

In pure culture studies, mirex and carbofuran failed to inhibit gelatin hydrolysis, starch hydrolysis, lipid hydrolysis, casein hydrolysis, chitin hydrolysis, glucose fermentation, H_2S production, nitrate reduction, ammonification and methane utilization. These concentrations also failed to inhibit methane utilization and nitrification by estuarine sediments. Ten ppm mirex and carbofuran did not inhibit the metabolic activity of estuarine pond water, but did inhibit primary productivity. Concentrations of 1000 ppm mirex and carbofuran did not effect the growth curves of bacterial cultures in broth.

Microcosm studies confirmed the lack of inhibition by mirex. A concentration of 1000 ppm did not significantly alter the mixed microbial populations or the microbial activities of estuarine sediments. On the other hand, the addition of carbofuran to sediments in concentrations of 100 ppm or greater caused an increase in the numbers of aerobic heterotrophic bacteria, but did not favor the development of any single type of bacteria. Further, the addition of carbofuran to sediments, in concentrations of 100 ppm or greater, significantly reduced the sediment dehydrogenase activity, the ability of the sediment microflora to mineralize glucose, the rate of oxygen utilization during glucose assimilation by sediment microorganisms, and the production of hydrogen sulfide by sediment bacteria.

Degradation products of mirex and carbofuran were shown to be far more toxic to microorganisms than were the parent compounds. Of the mirex degradation products, kepone and reduced kepone exhibited the greatest toxicity. The 3-hydroxycarbofuran was more toxic than either carbofuran or 3-ketocarbofuran.

Ultraviolet irradiation of moist mirex resulted in a bacteriologically toxic material.

SECTION VII

DISCUSSION

The following paragraphs describe briefly the rationale whereby the conclusions were drawn from this study.

Mirex is known to be very insoluble in water; also, it is quickly adsorbed onto particulate matter and ultimately settles to the bottom where it is not subject to photochemical degradation. It resists decomposition and is virtually inert under normal environmental conditions. Therefore in nature, in the aqueous environment, mirex will not remain long in the water column but may have an appreciable lifetime in the sediments.

While carbofuran is not readily adsorbed, it has been shown that more than 75% of the carbofuran in an aqueous environment disappears in a short time probably due to hydrolysis to the phenol. Therefore, buildup of this pesticide is not likely.

The only microbiological activity appreciably effected by either mirex or carbofuran at a level below 100 ppm was the inhibition of primary productivity. As a result of the low water solubility and the high rate of depletion by adsorption in the case of mirex and the rapid disappearance of carbofuran by other mechanisms, neither are expected to have any significant effect on primary productivity in the environment since the majority of the phytoplankton are in the aqueous phase, rather than in the sediments and most of the material would be attached to particulate matter prior to entrance into the water.

While 3-hydroxycarbofuran has been shown to be toxic in concentrations as low as 1 ppm, this derivative was not produced from carbofuran in the fate studies. Although degradation products of mirex have been shown to be toxic at concentrations of 1 ppm, they were not found in detectable quantities in the fate studies. In another study¹⁶ of mirex under environmental conditions, the yields of degradation products were extremely low. This low conversion, coupled with the probability that the degradation products may be more biodegradable than the parent compound, leads to the expectation that they will not cause problems in the environment.

Highly significant was the finding that purified mirex was not toxic to microorganisms while some batches of technical mirex and UV irradiated moist mirex were toxic. It may be concluded that other studies on the toxicity of mirex should be re-evaluated. This is especially true in view of the fact that one of the breakdown compounds studied in this investigation has the same retention time as mirex and because the concentrations of degradation products that would cause observable toxicity is such that they would not be detected using normal gas chromatographic procedures. All of these compounds are more polar than mirex and should have higher water solubility than mirex. These facts may be important factors in toxicity studies in water.

SECTION VIII

REFERENCES

1. EPA. 1972 Summary of Registered Agricultural Pesticide Chemical Uses. III-C.1-4.2, 1972.
2. Kennedy, M., M. Holliman and B. R. Layton. (Personal communication) Identification of the Major Thermal Product of the Insecticide Mirex, to be published J. Agr. Food Chem.
3. Alley, E. G., D. A. Dollar, B. R. Layton and J. P. Minyard, Jr. Photochemistry of Mirex. J. Agr. Food Chem. **21**(1):138-139, 1973.
4. Alley, E. G., B. R. Layton and J. P. Minyard, Jr. Identification of the Photoproducts of the Insecticides Mirex and Kepone. J. Agr. Food Chem. **22**(3):442-445, 1974.
5. Dilling, W. L., H. P. Braendling and E. T. McBee. Pentacyclodecane, Chemistry II. Tetrahedron **23**:1211-1224, 1967.
6. Metcalf, R. L., T. R. Fukato, C. Collins, K. Borck, S. A. El-Azie, R. Munoz and C. C. Cassil. Metabolism of 2,2-dimethyl-2,3-dihydrobenzofuranyl-7-N-Methylcarbamate (Furadan) in Plants, Insects, and Mammals. J. Agr. Food Chem. **16**:300, 1968.
7. American Public Health Association (APHA). Standard Methods for the Examination of Water and Wastewater. 13th ed. American Public Health Association, Inc., New York, 1971.
8. Cook, D. W. Metabolism of Carbohydrates by *Hydrogenomonas eutropha*. Ph.D. Dissertation, Mississippi State University, 1966.
9. Hutton, W. E. and C. E. ZoBell. The Occurrence and Characteristics of Methane-Oxidizing Bacteria in Marine Sediments. J. Bacteriol. **58**:463-473, 1949.
10. Brown, L. R., R. J. Strawinski and C. S. McCleskey. The Isolation and Characterization of *Methanomonas methanooxidans* (Brown and Strawinski). Can. J. Microbiol. **10**:792-799, 1964.
11. Mitchell, Ralph, S. Fogel and I. Chet. Bacterial Chemoreception: An Important Ecological Phenomenon Inhibited by Hydrocarbons. Water Res. **6**:1137-1140, 1972.
12. Harrison, M. J., R. T. Wright and R. Y. Morita. Method for Measuring Mineralization in Lake Sediments. Appl. Microbiol. **21**:698-702, 1971.
13. Lowry, O. J., N. J. Rosenbrough, H. L. Farr and B. J. Randall. Protein Measurements with Folin Phenol Reagent. J. Biol. Chem. **193**:265-275, 1951.

14. Pamatmat, M. M. and A. M. Bhagwat. Anaerobic Metabolisms in Lake Washington Sediments. *Limn. and Oc.* 18:611-623, 1973.
15. Anonymous. U. S. Dept. H.E.W. Washington, D.C. Pesticide Analytical Manual 1:211-212, 1973.
16. Ivie, G. W., H. W. Dorough and E. G. Alley. Photodecomposition of Mirex on Silica Gel Chromatoplates Exposed to Natural and Artificial Light. *J. Agr. Food Chem.* 22(6):933-935, 1974.